昭和32年7月9日 第三種郵便物認可
第50巻 第2号 昭和36年8月25日 発行
（毎月1回25日発行）
The Journal of Biochemistry

Vol. 50 No. 2 August 1961

THE JOURNAL OF BIOCHEMISTRY

EDITED FOR
THE JAPANESE BIOCHEMICAL SOCIETY

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BY
THE JAPANESE BIOCHEMICAL SOCIETY
The University of Tokyo, Tokyo

THE JOURNAL OF BIOCHEMISTRY was founded in 1922 by Prof. emeritus Dr. S. Kakiuchi and had been edited by him to volume 36 of the year of 1944. After the discontinuation during the War, this JOURNAL has been restarted by The Japanese Biochemical Society, following the serial number from volume 37 of the year of 1950.

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The Journal of Biochemistry, Vol. 50, No. 2, 1961

Preliminary Observations on the Distribution and Biosynthesis of Serine Ethanolamine Phosphodiester

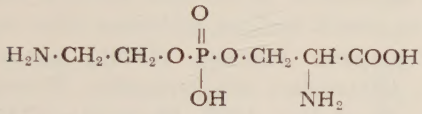
(Dedicated to Professor K. Kodama on the occasion of his 70th birthday)

By H. Rosenberg and A. H. Ennor

(*From the Department of Biochemistry, John Curtin School of Medical Research, Australian National University, Canberra, A. C. T., Australia*)

(Received for publication, June 7, 1961)

L-Serine ethanolamine phosphodiester (L-SEP) was discovered by Roberts and Lowe (*1*) in alcoholic extracts of river turtle muscle and was accorded the following structure



$$H_2N\cdot CH_2\cdot CH_2\cdot O\cdot \overset{\overset{O}{\|}}{\underset{\underset{OH}{|}}{P}}\cdot O\cdot CH_2\cdot \underset{\underset{NH_2}{|}}{CH}\cdot COOH$$

on the basis of degradation experiments. The serine component was shown (*1*) to possess the L-configuration and the structure above was subsequently confirmed by synthesis (*2*). L-SEP was also found in alligator and snake muscle (*3*) but was reported absent from mouse tumours and chick embryos (*1*). Attempts which have been made on the *in vitro* incorporation of isotopically labelled acetate and glycine into SEP were unsuccessful but a low level of incorporation of P^{32} was achieved into kidney SEP following the intracardiac injection of inorganic P^{32} into turtles (*4*).

The D-isomer of SEP was discovered in earthworms by Rosenberg and Ennor (*5*) in 1959 and was later isolated as a crystalline substance by the same group (*6*). In the earthworm this compound is the precursor of lombricine, to which it is converted by a transamidination reaction involving arginine (*7*). The interest of the present authors in SEP is mainly in the D-isomer and centres around the origin of D-serine and the mechanism of its incorporation together with that of ethanolamine and inorganic phosphate into the molecule. In this connection it should be mentioned that D-serine has been isolated from earthworms and that this enantiomorph is present in the free amino acid pool (*8*, *9*). However, it seemed worthwhile to determine the distribution of SEP in nature with a view to finding an animal more suited, in the first instance at least, to investigations on the biosynthetic pathways leading to its formation, than the earthworm.

This communication therefore presents in preliminary form the results of this, as yet incomplete, survey, and some results of the *in vivo* and *in vitro* incorporation of inorganic P^{32} into L-SEP.

EXPERIMENTAL

All animals used in the distribution studies were killed immediately before the experiment and the skeletal muscles taken for extraction. In the case of the smaller marine and terrestrial specimens the whole organism was extracted. Extracts were prepared by disintegration of the tissue in a Waring blendor in an equal volume of cold $1.5\,N$ $HClO_4$. The acid suspensions were filtered and adjusted to pH 7–7.5 by the addition of $10\,N$ KOH. When the amount of tissue taken was sufficient to permit isolation of SEP as a crystalline, chemically pure product, the subsequent procedure followed that previously described (*6*). If such a technique was precluded the SEP contained in the extract was identified by chromatographic means (*5*)—in such cases it was not possible to determine which isomer was present.

Radioactivity measurements and P estimations were made on samples recovered from chromatograms and wet-ashed in $10\,N$ H_2SO_4. The amount of SEP present was calculated from the P content of such

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The subject matter of this article was presented at an International Conference on Free Amino Acids, held at the City of Hope Medical Centre, Duarte, California, May 19–22, 1961.

samples which, although not yielding a precise quantitative result, is believed to give a figure ±10% of that actually present in the tissue examined.

RESULTS

From the results so far obtained it would seem that SEP is restricted to oligochaetes, where it is of the D-configuration, and to birds, amphibians, reptiles and fish, where it is exclusively the L-isomer (Table I).

Experiments were then carried out with a river turtle, lizard, chicken and a frog, which were injected intraperitoneally or intravenously with inorganic P^{32} and killed 1 hour later to determine if any isotope had been incorporated into SEP isolated from various organs. An appreciable degree of incorporation was observed only in the chicken and this animal (16 weeks old) was therefore used for subsequent experiments.

The probable site of synthesis of SEP in the 16-week old chicken has been determined by isolation of SEP from various organs some time after the intravenous injection of inorganic P^{32} and determination of the SEP content and specific radioactivity. There is an extremely wide variation between the organs examined in so far as their SEP content is concerned but the variation in the degree of isotopic labelling is much less variable (Table II). The results suggest that SEP is synthesized in the kidney but they do not exclude its formation in the small intestine.

Following this experiment a successful attempt was made to obtain isotope incorporation in a chicken kidney homogenate. This was prepared by homogenizing two chicken kidneys (15 g.) in 30 ml. of 0.05 *M* N-ethylmorpholine buffer, pH 7.2. The homogenate was filtered through a fine mesh nylon gauze to remove debris and divided into 3 parts to which various additions were made. The complete mixture (15 ml.) contained Mg^{++}, 100 μmoles; ethanolamine, 50 μmoles; serine, 50 μmoles; ADP, 50 μmoles; CTP, 5 μmoles, K-succinate, 100 μmoles; N-ethylmorpholine buffer, pH 7.2, 500 μmoles and variations from this are indicated in Table III.

The results indicate that an *in vitro* synthesis of SEP has been achieved and that this is an energy dependent reaction since a

TABLE I

Distribution and Identity of SEP in Animals

Group Examined	No. of Species	Result	Group Examined	No. of Species	Result
Protozoa	1	No trace of SEP detected	Pisces	5	L-SEP identified in one marine and one fresh water. Absent from 3 marine
Porifera	1				
Coelenterata	1				
Ctenophora	1				
Chaetognatha	1				
Platyhelminthes	1				
Nematoda	2		Aves[3]	3	L-SEP identified in one sp.
Annelida[1]	5				
Arthropoda	7		Amphibia	3	L-SEP identified in all.
Mollusca	9				
Echinodermata	5				
Urochorda	1		Reptilia	5	L-SEP identified in all.
Mammalia[2]	10				

[1] Excluding terrestrial oligochaetes in which D-SEP is known to be present (*5*, *6*).

[2] Includes representatives of monotremata, marsupialia and eutheria.

[3] SEP appears in the developing chicken embryo on the 13th day.

TABLE II

The Distribution and Specific Radioactivity of SEP in the 16 Week Old Chicken Following the Intravenous Administration of Inorganic P^{32}

Tissue	SEP	
	μmoles/g.	Specific Radioactivity c.p.m./μmole 70 min. after injection
Kidney	240	1150
Duodenum	190	202
Jejunum	190	328
Ileum	120	805
Pancreas	31	117
Spleen	11	310
Heart	10	515
Brain	6.3	274
Skeletal muscle	1.3	224
Liver	0.7	310
Plasma	0.3	1190
Erythrocytes	0.9	970

TABLE III

In Vitro Incorporation of Inorganic P^{32} into SEP in Chicken Kidney Homogenates

(Incubation 2 hours at 37°C with shaking—conditions as in text)

Condition	Specific Radioactivity c.p.m./μmole
Complete mixture	805
Complete mixture+ 2 : 4-dinitrophenol (3×10^{-4} *M*)	0
Complete mixture less CTP	490

complete suppression of incorporation of P^{32} was achieved in the presence of dinitrophenol. The diminished degree of incorporation when CTP was omitted suggests that this nucleotide may be involved in the biosynthetic reaction which lead to SEP formation.

Some experiments have been carried out with homogenates prepared from liver and small intestine under conditions which have permitted P^{32} incorporation in kidney homogenates. In no case has any incorporation of P^{32} been achieved.

DISCUSSION

Although the search for SEP in various animals is far from complete, it would seem that the L-isomer is confined to birds, fish, reptiles and amphibians. This peculiar distribution and the close evolutionary relation which these groups bear to each other suggest that the compound may have some evolutionary significance.

The results leave little doubt that the kidney is the sole site of synthesis in the chicken and although they do not prove this possibility, the failure to achieve isotope incorporation into L-SEP with preparations from the small intestine and liver certainly strengthens it. The high specific radioactivity of SEP in plasma suggests that SEP, once synthesized in the kidney, is then transported from this organ and distributed through the body perhaps to perform some specific function in the small intestine where its concentration is relatively high.

The preliminary experiments with chicken kidney homogenate prove quite conclusively that L-SEP is formed from small molecular weight precursors and the inhibitory effect of dinitrophenol shows, as would be anticipated, that the biosynthesis is energy dependent. It is not possible at this stage to be dogmatic about other requirements of the synthetic system, although the omission of CTP did result in about a 50% diminution of P^{32} incorporation. This suggests that CTP may be involved in the synthesis perhaps in somewhat the same way as in the synthesis of phosphatidylserine and phosphatidylethanolamine (*10*, *11*). It is anticipated that experiments now in progress will enable the complete biosynthetic pathway to be defined in detail in the near future.

It is a pleasure to acknowledge our indebtedness to Miss Miriam Morgan, A.B., M.A., a Fulbright Scholar from the United States who carried out the major part of the survey of SEP distribution reported here. Our thanks are also due to Mr. Brian Thorpe for skilled technical assistance.

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The Journal of Biochemistry, Vol. 50, No. 2, 1961

Studies on Nitro-Reducing Enzymes of Swine Liver

Properties and Cofactor Requirements of Nitro- and Nitrosoreductases**

By Shigeru Otsuka*

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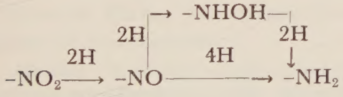
(Received for publication, October 13, 1960)

Aromatic nitro compounds have been shown to be reduced to the corresponding arylamines in mammalian tissues (*1–3*), especially in liver (*4–8*), and this reduction has been attributed to the action of an enzyme system called "nitroreductase". Reduced pyridine nucleotides have been found to act as hydrogen donors for the nitro-reducing reaction. Several workers have suggested that flavin nucleotides and heavy metals are involved in the reduction mechanisms (*4–8*) and Westerfeld *et al.* (*9*) have postulated molybdenum as an essential component of nitroreductase based on the finding that the enzyme level in rat liver was significantly lowered in molybdenum deficiency.

Chemically speaking, the reduction of a nitro group to amino group is a process involving the transfer of six atoms of hydrogen and therefore most probably consists of the following two or three intermediary steps:



$$-NO_2 \xrightarrow{2H} -NO \xrightarrow{4H} -NH_2$$

$$-NO \xrightarrow{2H} -NHOH \xrightarrow{2H} -NH_2$$

Little investigations have, however, been made concerning the intermediary steps involved in the nitroreductase reaction, except for the study of Yamashina (*10*) who showed that the reduction of aromatic nitro compounds to arylamines by an enzyme preparation from a halotolerant bacterium takes place through the nitroso stage. He has, however, not studied whether different enzymes are concerned in these intermediary steps.

In the course of purification of the liver nitroreductase system by ion-exchange chromatography, the author has succeeded in separating the system into two protein fractions, one catalyzing only the reduction of nitro to nitroso and the other only the step from nitroso to amino. The purpose of the present paper is to describe the separation and purification of the two enzymes and report some of their enzymatic properties.

EXPERIMENTAL

Preparation of Crude Enzyme—One kg. of fresh swine liver was homogenized with 2 liters of cold water, and the homogenate was centrifuged at 10,000 r.p.m. for 20 minutes. To 1 liter of the supernatant adjusted, if necessary, to pH 6.5 with dilute NaOH was added 250 g. of solid ammonium sulfate, and the resulting precipitate was removed by centrifugation. 70 g. of ammonium sulfate was further added to the supernatant, and the precipitate containing nitro-reducing enzymes was collected by centrifugation and dissolved in 100 ml. of water. The solution was dialyzed and designated as Fraction A, which was used as the enzyme in preliminary tests.

Purification of Nitroreductase—Two hundred ml. of Fraction A was dialyzed against 0.006 *M* sodium citrate, pH 7.8, for 36 hours at 5°C and then applied to a DEAE-cellulose column (1.7×27 cm.) which had previously been bufferized with 0.006 *M* sodium citrate. The column was washed with 0.006 *M* citrate at 4°C. Under these conditions, only nitroreductase (NOR) was adsorbed to the column and all of the nitrosoreductase (NSR) activity rapidly passed through the column. The NOR adsorbed was then eluted from the column with sodium citrate; the concentration of which being gradiently increased from 0.006 *M* to 0.08 *M*. The effluents containing NOR were combined, dialyzed against 0.006 *M* sodium citrate, and again applied to a small column of DEAE-cellulose bufferized with the same citrate solution. The gradient elution was carried out as before and the fractions containing purified NOR were collected. The enzyme was finally precipitated from the solution by solid ammonium sulfate between 40 and 45% saturation. The purity and yield of the enzyme at each step of purification are summarized in Table I.

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** The following abbreviations will be used: diphosphopyridine nucleotide (DPN^+), reduced diphosphopyridine nucleotide (DPNH), triphosphopyridine nucleotide (TPN^+), reduced triphosphopyridine nucleotide (TPNH), flavin-adenine dinucleotide (FAD), flavin mononucleotide (FMN), *p*-nitrophenol (NP), *p*-nitrosophenol (NSP), *p*-aminophenol (AP), *o*-nitrobenzoic acid (NBA), *o*-nitrosobenzoic acid (NSBA), nitroreductase (NOR), nitrosoreductase (NSR), alcohol dehydrogenase (ADH), ethylenediamine tetraacetate (EDTA).

TABLE I

Purification of Nitro- and Nitrosoreductases

	Reduction of nitro1)		Reduction of nitroso	
	Aminophenol formed per mg. prot.	Recovery2)	Aminophenol formed per mg. prot.	Recovery2)
	mμmole		mμmole	
Extract	520	100	3,670	100
First ammonium sulfate precipitate	1,200	38.2	14,270	32.9
Filtrate from DEAE-cellulose column	0	—	71,000	30.3
First eluate with 0.08 *M* sodium citrate	5,000	13.7	—	—
Second eluate with 0.08 *M* sodium citrate	8,220	6.5	—	—
Second ammonium sulfate precipitate	8,510	6.2	—	—
Eluate with 0.006 *M* sodium citrate	0	—	123,300	13.1

1) Excess amount of NSR preparation was added to incubation media.
2) Relative units taking Extract as 100.

Purification of Nitrosoreductase—The solution containing nitrosoreductase (NSR) which had passed through the DEAE-cellulose column by washing with 0.006 *M* citrate was dialyzed against 0.001 *M* tris(hydroxymethyl)aminomethane (Tris) solution (free base) in the cold. The dialyzed solution was applied to a DEAE-cellulose column (1.7×23 cm.) which had been converted to the OH-type with *N* NaOH and then treated with 0.001 *M* Tris solution.

The enzyme was then eluted from the column with 0.001 *M* Tris solution containing sodium citrate of gradiently increasing concentration (0 to 0.015 *M*); the flow rate being 25 ml. per hour. The fractions containing NSR were combined and the enzyme was concentrated by ammonium sulfate (between 35 and 45% saturation). The progress of purification is recorded in Table I.

Assay for Nitroreductase Activity (*Nitro to Nitroso*)—The NOR activity was estimated by measuring the formation of *p*-nitrosophenol or *p*-aminophenol; excess of NSR being added in the reaction mixture when *p*-aminophenol was the product to be determined. The reaction mixture usually contained 0.3 ml. enzyme, 2 μmoles *p*-nitrophenol, "DPNH-generating system" (0.004 mole ethyl alcohol, 1 mg. DPN, and approximately 0.1 mg. yeast alcohol dehydrogenase), and 0.3 ml. NOR solution, and the total volume was made up to 2.5 ml. with 0.1 *M* phosphate buffer, pH 7.8. The reaction was run in a modified Thunberg tube at 37.5°C in a nitrogen atmosphere. After incubating for 1 hour, the reaction was stopped by heating the tube in a boiling water bath for 4 minutes, and the resulting precipitate was removed by centrifugation. The clear supernatant thus obtained was then analyzed for the phenols.

In cases where the effects of inhibitors etc. were to be studied, approximately 3.1 μmoles DPNH was added in place of the "DPNH-generating system" to avoid the interferences of alcohol dehydrogenase activity by the added substances.

Assay for Nitrosoreductase Activity (*Nitroso to Amino*)—The NSR activity was estimated by measuring the amount of *p*-aminophenol formed from *p*-nitrosophenol. The reaction mixture consisted of 0.3 ml. NSR solution, 5 μmoles *p*-nitrosophenol, "DPNH-generating system" (the same as that used in the NOR assay), and 0.1 *M* phosphate buffer, pH 7.8, to make the final volume to 2.5 ml. The reaction was carried out for 5 minutes at 37.5°C in a nitrogen atmosphere and stopped by heating the mixture for 4 minutes in a boiling water bath. After centrifugation, the supernatant was subjected to the analysis for *p*-aminophenol.

Analytical Methods

p-Nitrophenol—To an aliquot of the sample was added an equal volume of *N* NaOH, and the yellow color due to *p*-nitrophenol was measured in a spectrophotometer at 420 mμ.

p-Nitrosophenol—To 3 ml. of the sample was added 0.2 ml. of aqueous solution of 0.01% sodium pentacyanoammine ferroate. After 30 minutes or more, the intensity of the olive green color was colorimetrically estimated at 690 mμ.

p-Aminophenol—To 2 ml. of the sample, 0.3 ml. *N* Na_2CO_3, 0.3 ml. 5% phenol, and 0.2 ml. of 0.2% sodium ferricyanide were added successively with vigorous shaking after each addition. After 15 minutes, the deep blue color of indophenol dye formed was measured at 640 mμ.

Anthranilic Acid—The incubation mixture was deproteinized by the addition of an equal volume of 10% trichloracetic acid. Into 2 ml. of the filtrate, 0.2 ml. of 0.2% $NaNO_2$, 0.2 ml. of 1% ammonium sulfamate, and 0.1 ml. of 0.5% 1-N-naphthylethylenediamine hydrochloride were added at intervals of 10 minutes. The pink color of diazo dye formed was measured in the spectrophotometer at 535 mμ.

Preparation of Substrates—*p*-Nitrosophenol was synthesized by the nitrosation of phenol by the method of Yamaguchi (*11*). *o*-Nitrosobenzoic acid was synthesized by the photochemical intermolecular conversion of *o*-nitrobenzaldehyde according to the method of Ciamician and Silber (*12*). The *o*-substituted benzoic acids were used as substrates in place of *p*-substituted phenols in the studies on the behavior of flavin nucleotides, since the colorimetric measurement of flavins at 455 mμ was interfered with by the absorption of *p*-nitrophenol.

Pyridine Nucleotides—Diphosphopyridine nucleotide of approximately 60 per cent purity, prepared from the baker's yeast by a modification of the method of Le Page (*13*) was kindly supplied from Prof. Okunuki of Osaka University. The nucleotide of purity of 95 per cent was purchased from the Nutritional Biochemicals Corporation Ltd. and used in some of the experiments. Triphosphopyridine nucleotide of purity of 95 per cent was purchased from the Nutritional Biochemicals Corporation Ltd. Reduced diphosphopyridine nucleotide was prepared enzymatically by the method of Rafter and Colowick (*14*).

Alcohol dehydrogenase was prepared by the method of Racker (*15*) from baker's yeast.

Glucose-6-phosphate dehydrogenase was prepared from brewer's yeast by the method of Kornberg and Horecker (*16*).

Glucose-6-phosphate prepared by the method of Horecker *et al.* (*17*) was kindly supplied from Prof. Sato of the Institute for Protein Research, Osaka University.

"TPNH-generating system", which consisted of 0.004 mole glucose-6-phosphate, 1 mg. TPN^+, 10^{-4} mole $MgCl_2$ and approximately 2 mg. of glucose-6-phosphate dehydrogenase, was used for the studies on the specificities of the enzymes for hydrogen donors. The capacity of generating TPNH was controlled by the amount of the added enzyme so as to produce the same amount of the reduced pyridine nucleotide as in the case of the above "DPNH-generating system".

RESULTS

Properties of Crude Enzyme—Preliminary characterizations of the nitro-reducing system were made using the crude enzyme preparation obtained by ammonium sulfate fractionation (Fraction A). As already mentioned, this fraction contained both NOR and NSR.

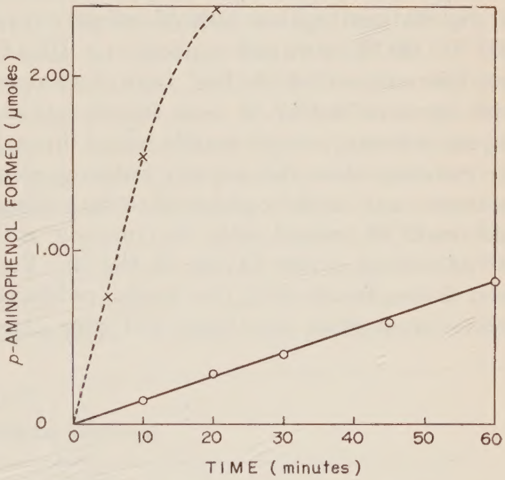


FIG. 1. Reduction of *p*-nitro- and *p*-nitrosophenols by a preparation from swine liver (Fraction A).

0.3 ml. enzyme solution, 0.004 mole ethyl alcohol, 1 mg. DPN^+, approximately 0.1 mg. of yeast alcohol dehydrogenase, and 2 μmoles *p*-nitro- or *p*-nitrosophenols (total volume; 2.5 ml.) were incubated under nitrogen at 37.5°C.

—○— *p*-Nitrophenol
--×-- *p*-Nitrosophenol

Fig. 1 shows that Fraction A catalyzed the reduction of *p*-nitrosophenol much more rapidly than that of *p*-nitrophenol. This suggests that the rate-limiting step in the whole reduction process in liver is the first step, *i.e.*, from nitro to nitroso.

In order to see if flavin is involved in the reduction mechanism, the enzyme was treated with saturated calcium chloride at pH 5.5 for 1 hour and then dialyzed. It was thus found that the activity of the dialyzed solution could be slightly accelerated by the addition of flavin mononucleotides.

An attempt to remove metal components from the enzyme by treatment with KCN according to the method of Nicholas *et al.* (*18*) resulted in the decrease of the activity by about 25 per cent. Fe^{++}, Fe^{+++}, Mn^{++}, MoO_4^{--} or WO_4^{--} were, however, all unable to detectably restore the activity when these metal ions (in a concentration of $10^{-4}M$) were incubated for 20 minutes with the treated enzyme.

Separation of the Enzymes—When Fraction A was dialyzed against 0.006 *M* sodium citrate (pH 7.8) for 24 hours and applied to a DEAE-cellulose column which had been bufferized with the same buffer, it was found that the nitroso-reducing activity readily passed through the column, while the activity reducing nitro to nitroso was strongly adsorbed to the column and could be eluted only by treating with 0.03 *M* sodium citrate (Table II, Fig. 2). Further, it was found that the whole reduction process from nitro to amino did take place only when the two fractions were combined, whereas the complete reduction was not observed by the addition of heat-inactivated preparation of either one of the enzymes to the other. Hence it can be concluded that the complementary effect of the two fractions was not due to the supply of any low-molecular cofactors which would have been removed from apo-proteins by ion exchanger.

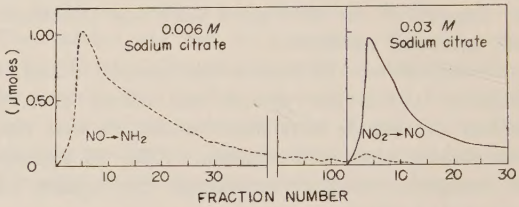


FIG. 2. Separation of nitro- and nitrosoreductases with DEAE-cellulose column.
—— Activity of reduction from nitro to nitroso.
- - - Activity of reduction from nitroso to amino.
Values show the amounts of *p*-nitroso- or *p*-aminophenols formed in μmoles.

However, as seen in Table II, only one third of the amount of *p*-nitrosophenol expected from the disappearance of *p*-nitrophenol was formed by the action of nitro-reducing fraction (Fraction II). At the same time, a small amount of *p*-aminophenol was determined. The sum of the amounts of these two phenols was found to be still far less from the corresponding amount of *p*-nitrophenol disappeared.

TABLE II

Separation of Nitro- and Nitrosoreductases

	Reduction of *p*-nitrophenol			Reduction of *p*-nitrosophenol	
	Nitrophenol disappeared	Nitrosophenol formed	Aminophenol formed	Nitrosophenol disappeared	Aminophenol formed
	μmole	μmole	μmole	μmole	μmole
Fraction II	0.208	0.070	0.050	0.004	0.004
Fraction I	0	0	0	0.301	0.229
F. II + F. I	0.242	—	0.237	0.406	0.380
Boiled F. II1)	0	0	0	0.004	0.008
Boiled F. I1)	0	0	0	0.004	0.008
F. I + boiled F. II	0	—	—	—	0.302
F. II + boiled F. I	0.208	0.063	0.061	—	0.002

1) Kept in a boiling water bath for 4 minutes.

A possible explanation for these facts is that a considerable amount of *p*-nitrosophenol formed by the action of Fraction II has been converted, either by condensation with other phenols or by decomposition, into certain unidentified substances which are inactive in the color reaction with the pentacyanoammine ferroate reagent.

Identification of Reaction Products by Paper Chromatography—The reaction products of the two enzyme reactions were further confirmed by paper chromatography of ether extracts of the reaction mixtures obtained from three reaction systems: (A) Fraction I, Fraction II and *p*-nitrophenol, (B) Fraction II and *p*-nitrophenol, and (C) Fraction I and *p*-nitrosophenol (Fig. 3).

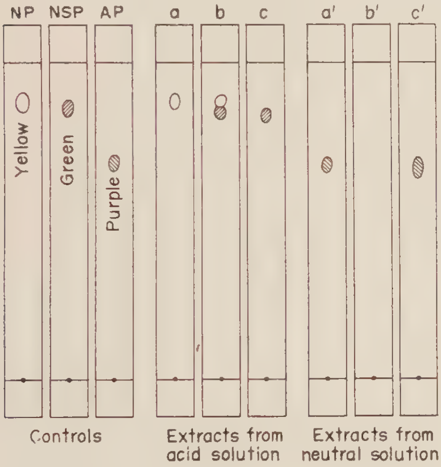


FIG. 3. Identification of reduction products. (A) 1 ml. of Fraction I, 1 ml. of Fraction II, and 5 μmoles of *p*-nitrophenol, (B) 1 ml. of Fraction II and 5 μmoles of *p*-nitrophenol, or (C) 1 ml. of Fraction I and 5 μmoles of *p*-nitrosophenol, were incubated anaerobically with the "DPNH-generating system" (five-fold the amounts of usual experiments were used), for 1 hour, at 37.5°C. Reaction was stopped by the addition of the equal volume of 4 *N* HCl, and precipitated protein was filtered off. *p*-Nitro- and *p*-nitrosophenols were extracted with peroxide-free ether from the acid filtrates for 10 hours by the use of Soxhlet extractors. The acid solutions which contained *p*-aminophenol remaining were now neutralized with solid Na_2CO_3 and the extractions were carried out for additional 10 hours as above.

The extracts from acid filtrates (a, b, and c) and from neutralized solutions (a', b', and c') were, after the evaporation of ether, subjected to paper chromatography using *n*-butanol, acetic acid and water (4 : 1 : 5) as the solvents. The phenols were identified by a comparison of their R_f values and the colors developed with those of pure reference compounds, after spraying successively 0.5 *N* NaOH and 0.02% sodium pentacyanoammine ferroate.

As can be seen in Fig. 3, (a and a'), the completion of the reduction of *p*-nitrophenol to *p*-aminophenol is attained only by the consecutive actions of the two fractions, and the absence of nitroso-reducing fraction (Fraction I) results in the accumulation of *p*-nitrosophenol (b and b').

It will be evident from these results that the roles of the two different liver enzymes should be distinguished from each other, that is, the one is specific for nitro group and the other for nitroso group.

Absorption Spectra—Purified NSR was almost colorless even in highly concentrated solution. In fact, the absorption spectrum of NSR (Fig. 4) showed no maxima except for a peak at 280 mμ. Purified NOR, on the other hand, was reddish yellow in color suggesting the presence of a flavin component. As can be seen from Fig. 4, however, its absorption spectrum possesses no characteristic flavin band at 460 mμ. This fact, together with the marked stimulation of NOR activity of added FAD (Table V), suggests that the flavin component had been detached from the enzyme during the purification. The

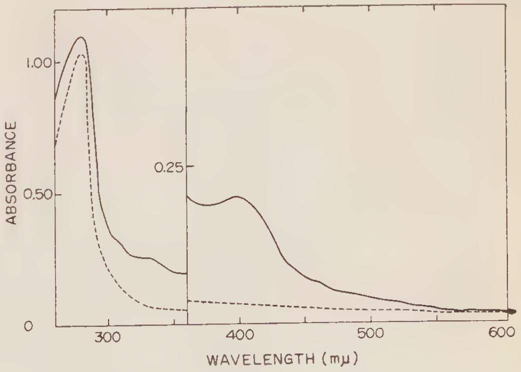


FIG. 4. Absorption spectra of nitro- and nitrosoreductase preparations.
—— Nitroreductase, - - - Nitrosoreductase.

nature of a broad band at around $400\,\mathrm{m}\mu$ observed in the NOR spectrum is not yet clear.

Reaction Velocities—As mentioned already, the crude enzyme (Fraction A) catalyzed the reduction of *p*-nitrosophenol to *p*-aminophenol much more rapidly than that of *p*-nitrophenol. This observation was now confirmed with purified preparations. Thus, the nitroso to amino reduction catalyzed by purified NSR was found to be 15 times as rapid as the nitro to nitroso reduction by purified NOR on the basis of the same protein content.

Honda, Shimoda and Otsuka (*8*) have reported previously that the nitro to amino reduction by a liver homogenate system is inhibited under aerobic conditions. In agreement with this finding, it was found that purified liver NOR is sensitive to atmospheric oxygen. Aerobiosis had, however, no inhibitory action at all toward the activity of purified NSR.

Effect of pH—Fig. 5 shows the effect of pH on both NOR and NSR. It will be seen that both enzymes acted maximally at pH 7.8. The pH curves were almost the same irrespective of the buffer components examined.

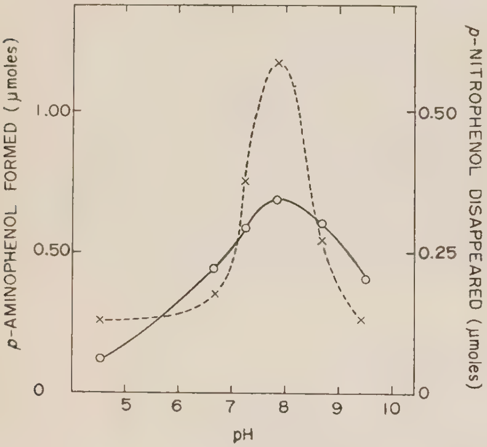


FIG. 5. pH-Dependences of nitro- and nitroso-reductase activities.

Incubation mixture contained 0.3 ml. DPNH, 5 μmoles *p*-nitro- or *p*-nitrosophenols and 0.3 ml. NOR or NSR preparations (total volume was made up to 2.5 ml. with 0.1 N phosphate buffers of various pH) for 1 hour in nitrogen for estimation of NOR activity, and for 5 minutes for NSR activity, at 37.5°C. —○— Nitroreductase activity (*p*-nitrophenol disappeared), --×-- Nitrosoreductase activity (*p*-aminophenol formed).

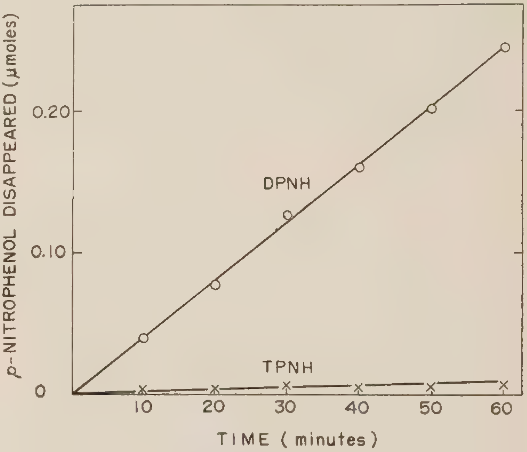


FIG. 6. Specificity of nitroreductase for reduced pyridine nucleotides.

p-Nitrophenol (1 μmole) and nitroreductase preparation (0.3 ml.) were incubated with the "DPNH-" or the "TPNH-generating systems" (described in "Experimental") for 1 hour in nitrogen at 37.5°C. Total volume, 2.5 ml.

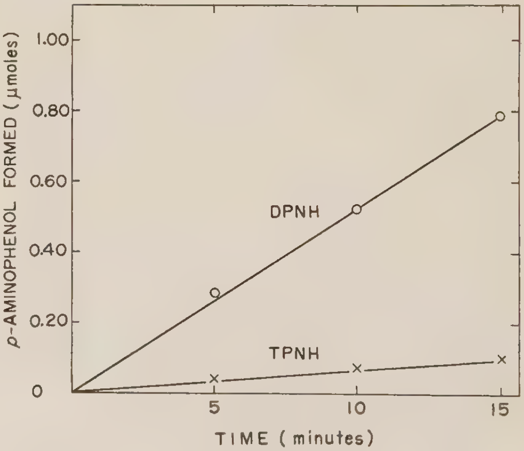


FIG. 7. Specificity of nitrosoreductase for reduced pyridine nucleotides.

p-Nitrosophenol (1 μmole) and nitrosoreductase preparation (0.3 ml.) were incubated with the "DPNH-" or the "TPNH-generating systems" (described in "Experimental") for 5 minutes at 37.5°C. Total volume, 2.5 ml.

Specificity for Hydrogen Donors—As can be seen from Figs. 6 and 7, DPNH was shown to act as an efficient hydrogen donor for both NOR and NSR. TPNH was, however, proved to be a very poor donor; its efficiency being less than 10 per cent of that of DPNH for both enzymes. Attempts to study the stoichiometry of the DPNH-nitro and -nitroso reactions by following the decrease in optical density at 340 mμ were not successful owing to intensive absorptions in the ultraviolet region caused by the substrates and products.

TABLE III

Effect of Inhibitors on Nitro- and Nitrosoreductases

Inhibitors	Concentration	Inhibition of nitroreductase	Inhibition of nitrosoreductase
	M	per cent	per cent
NaCN	5×10^{-2}	71.2	67.7
α, α'-Dipyridyl	10^{-2}	75.8	44.3
EDTA	〃	79.2	38.5
Thiourea	〃	13.9	—
Hydroxylamine	〃	—	67.0
Oxalate	〃	—	34.3
Semicarbazide	〃	—	0
Sodium azide	〃	—	28.7
Oxine	5×10^{-3}	74.7	—
$AgNO_3$	10^{-3}	79.4	82.7
Iodoacetic acid	5×10^{-3}	74.7	34.3
Atebrin	10^{-2}	32.0	26.0
Quinine	5×10^{-3}	31.5	29.0

DPNH was used as hydrogen donor in place of "DPNH-generating system".

Effects of Inhibitors—The effects of various inhibitors on the two enzymes are summarized in Table III. The fact that both enzymes are strongly inhibited by reagents capable of binding heavy metal ions (KCN, α,α'-dipyridyl, etc.) suggests the participation of certain heavy metal ions in the reduction mechanisms. In agreement with the result obtained with bacterial nitroreductase (*10*), thiourea even at a concentration of $10^{-2}M$ scarcely inhibited both enzymes.

The inhibitions caused by atebrin and quinine are to be noted, since these compounds are believed to compete with flavin nucleotides. Both of the reductases were appreciably inhibited by sulfhydryl reagents such as $AgNO_3$ and iodoacetate indicating the essential role of sulfhydryl group in both reductase reactions. The inhibition of NSR by iodoacetate was, however, considerably smaller than that of NOR.

Effects of Metal Ions—Since inhibition studies suggested the involvement of metal ions in the activities of reductase enzymes, the effects of several metal ions on both NOR and NSR were examined. As recorded in Table IV, however, all of the metal ions tested

TABLE IV

Effects of Metal Ions on Nitro- and Nitrosoreductases

Metal ion (10^{-4} *M*)	Nitroreductase (*p*-nitrosophenol formed)	Nitrosoreductase (*p*-aminophenol formed)
	μmole	μ mole
Nil	0.244	0.248
$FeCl_3$	0.223	0.298
$CuCl_2$	0.223	0.026
$MnCl_2$	0.212	0.234
$ZnCl_2$	0.257	0.237
Na_2MoO_4	0.269	0.287
Na_2WO_4	0.223	0.292

DPNH was added to the reaction medium in place of the "DPNH-generating system".

showed no stimulating actions. Only molybdate seems to cause slight acceleration of the activities of both enzymes. This is of some interest in view of the finding reported by Westerfeld (*9*) that molybdenum deficiency in rats causes a decrease in hepatic level of nitroreducing activity.

Roles of Flavin Nucleotides—The effects of added flavin nucleotides on purified NOR and NSR were studied. It will be seen from Table V that the NOR activity was greatly stimulated by FAD, whereas no effect was observed with FMN. The activity of NSR, on the other hand, was not affected by either of the nucleotides. It appears, therefore, likely that NOR specifically requires FAD for its activity and the purified enzyme has lost most of its FAD during preparation. It does not seem probable that NSR is a flavin enzyme.

TABLE V

Effects of Flavin Nucleotides on Nitro- and Nitrosoreductases

Supplement ($10^{-4}M$)	Nitroreductase (*p*-nitrosophenol formed)	Nitrosoreductase (*p*-aminophenol formed)
	µmole	µmole
Nil	0.244	0.248
FMN	0.235	0.244
FAD	0.650	0.252

DPNH was used as hydrogen donor in place of "DPNH-generating system".

The roles of flavins in the reductases were further studied by spectrophotometrically following the oxidoreduction of added flavin nucleotides at 455 mµ. As illustrated in Fig. 8, it was shown that both FAD and FMN can be reduced by NOR if a DPNH-generating system is present in the reaction mixture. The leuco form of FAD thus produced could be reoxidized by the addition of *o*-nitrobenzoate, whereas no reoxidation of leuco FMN took place by the addition of the nitro compound. It only inhibited further reduction of FMN. These findings provide another support to the conclusion that FAD is actually involved in the NOR activity. Although similar experiments were performed with NSR, neither reduction nor reoxidation of flavin nucleotides could be observed in agreement with the postulated non-flavoprotein nature of NSR.

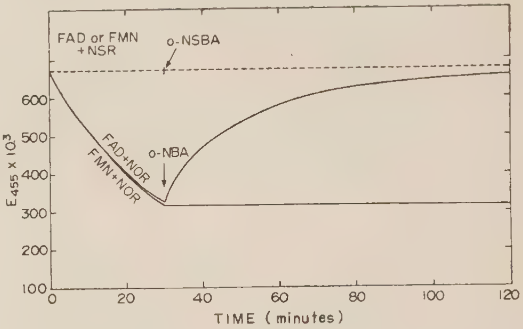


FIG. 8. Reduction and reoxidation of flavin nucleotides in the nitro- and nitrosoreductase systems.

Incubation medium contained the "DPNH-generating system", 3.6 mg. of NOR or NSR, and 2×10^{-4} moles of the flavin nucleotides (total volume was made up to 4 ml. with 0.1 *N* phosphate buffer, pH 7.8). 4 µmoles of *o*-nitro- or *o*-nitrosobenzoates (NBA or NSBA) were added at the time indicated by the arrows.

Incubations were made using modified Thunberg tubes and absorptions at 445 mµ were followed in a spectrophotometer *in vacuo* at 37°C.

DICUSSION

Villanueva (*20*) has recently described the isolation from *Nocardia* species of an ultracentrifugally homogeneous nitroreductase preparation and reported that the purified enzyme is capable of catalyzing the reduction of nitro compounds to corresponding amines. It is therefore likely that a single protein is responsible for the overall reduction from the nitro to amino stages at least in this microbial system.

The results reported in this paper, however, clearly show that the liver nitro-reducing system can be separated into two enzyme fractions, *i.e.* nitro- and nitrosoreductases, specific for the nitro to nitroso and nitroso to amino steps respectively. Although the separated liver enzymes have not yet been so highly purified as the *Nocardia* enzyme, it is certain that the reduction process in liver consists of at least two steps catalyzed by specific enzymes.

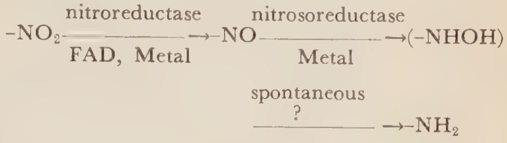
Yamashina (*10*) and Egami *et al.* (*19*) were the first to study the intermediary steps involved in the enzymatic reduction of nitro groups. Using a preparation obtained from a halotolerant bacterium, these workers suggested that three different enzymes, *i.e.* nitro-, nitroso- and hydroxylamino-reductases, are present in the preparation, although they did not attempt to separate these enzymes. Yamashina (*10*) also provided evidence indicating that nitrosoreductase alone is capable of reducing nitroso groups to the amino stage without the aid of "hydroxylamino-reductase".

Since a hydroxylamino group has been shown to be non-enzymatically reduced to an amino group by reduced pyridine nucleotides and by ferrous ion (Otsuka, unpublished observation), it may be safely concluded that, if a nitroso compound is reduced in liver to the hydroxylamino compound by the action of nitrosoreductase, the latter can be further reduced nonenzymatically to the amino stage. These facts strongly suggest that the two reductases separated in the present study would be sufficient to account for the overall reduction from nitro to amino in liver.

Liver nitroreductase seems to be a flavoprotein specifically requiring FAD for its activity, whereas any flavin does not seem to be involved in the nitrosoreductase activity. It is interesting to note that few enzymes have so far reported to oxidize DPNH without the participation of flavin nucleotides. FMN was found to be reduced by DPNH in the presence of NOR, but it could not be reoxidized by the addition of a nitro compound in contrast to reduced FAD. The FMN reduction may be attributed to the contamination of a DPNH-FMN reductase in the NOR preparation employed.

It seems fairly certain from inhibition studies that both reductases contain metal ions as the essential components. The nature of the metals involved has, however, not yet been elucidated.

Most of the data so far discussed may be explained by the following scheme of nitro-reduction:



$$-NO_2 \xrightarrow[\text{FAD, Metal}]{\text{nitroreductase}} -NO \xrightarrow[\text{Metal}]{\text{nitrosoreductase}} (-NHOH) \xrightarrow{\overset{\text{spontaneous}}{?}} -NH_2$$

SUMMARY

1. Two nitroreductase enzymes of the liver which are responsible for the reductions of nitro and nitroso groups respectively, were isolated separately and purified by the aid of DEAE-cellulose.

2. Several properties of the two enzymes have been studied; Both enzymes are active at pH 7.8. DPNH is about ten times active than TPNH as the hydrogen donor for both of the enzyme reactions. Nitroreductase is inhibited by oxygen, whereas nitrosoreductase is quite insensitive to oxygen. Both of the enzyme actions are inhibited by several metal-chelating agents, suggesting that they are both metallo-proteins. The enzymes are considered to have the active sulfhydryl groups from the inhibitory studies.

3. Nitroreductase was found to be a flavoprotein which specifically utilize flavin-adenine dinucleotide as hydrogen carrier, whereas the flavin nucleotides are considered not to be necessary for the reduction of nitrosophenol by the nitrosoreductase.

The author wishes to express many thanks to Prof. K. Honda for his kind guidance throughout this work.

The author's thanks are also due to Prof. K. Okunuki, Prof. S. Akabori of Osaka University, and Prof. R. Sato of the Institute for Protein Research, Osaka University, for their excellent advices and encouragements.

The author is further indebted to Prof. K. Narita of the Institute for Protein Research for his generous gifts of the ion exchangers.

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The Journal of Biochemistry, Vol. 50, No. 2, 1961

A Limited Proteolysis of Native Ovalbumin with a Proteinase from *Streptomyces griseus**

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(Received for publication, December 15, 1960)

It has been well established by Ottesen and Linderstrøm-Lang (*1*, *2*) that the transformation of ovalbumin into plakalbumin is a limited proteolysis catalyzed by subtilisin, a proteinase from *B. subtilis* (*3*, *4*). The proteolysis, as being a conversion of a native protein into another native protein and small peptide fragment(s) (*5*, *6*) seems to be similar to tryptic activation of zymogens in their reaction types (*7*, *8*).

The activation of zymogens, however, is catalyzed not only by trypsin, but also other proteinases (*9–12*) which are able to split the lysyl and arginyl linkages in an unfolded chain. Thus subtilisin catalyzes the activation of chymotrypsinogen (*9*), and a proteinase from *Aspergillus oryzae* catalyzes the split of Val-$(Asp)_4$-Lys-fragment from the N-terminal part of trypsinogen, giving active trypsin (*10*, *11*). The fact seems to suggest that the conversion of ovalbumin to plakalbumin or similar proteolysis may also be catalyzed by a proteinase other than subtilisin, if the enzyme acts only on the unfolded peptide chain part with a substrate specificity similar to (or wider than) that of subtilisin.

Recently, however, it was reported by Haruna (*13*) that a proteinase obtained from another strain of *B. subtilis* (Nagase (*14*, *15*)) catalyzed a limited proteolysis of ovalbumin, which was quite different from plakalbumin-formation in the reaction type. Thus about twenty amino acid residues in ovalbumin were liberated as three different oligopeptides, instead of Glu-Ala-Gly-Val-Asp and Ala-Ala in plakalbumin formation (*16*), while the protein moiety was obtained as another crystalline protein (H-protein).

In connection to his paper, we wish to report that a proteinase obtained from *Streptomyces griseus* (*17*, *18*) catalyzed a conversion of ovalbumin into plakalbumin-like protein, Ala-Ala and Ala-Gly-Val-Asp, although the *Actinomyces* proteinase catalyzed the hydrolysis of simple symthetic peptides with a substrate specificity fairly different from that of the above-mentioned bacterial proteinase (*19*, see also *3*).

EXPERIMENTALS

Materials—Crystalline ovalbumin was prepared from chicken egg white with the use of sodium sulfate at 30°C, according to the method of Kekwick and Cannan (*20*). The crystals, after twice recrystallisation and dialysis, were lyophilized. Analysis with the usual 1-fluoro-2, 4-dinitrobenzene method indicated that the free N-terminals derived from other contaminated protein, if it were present, were not more than 0.02 moles per 5×10^4 g. (*21*).

Streptomyces griseus proteinase used was a commercial preparation, "Pronase", from Kaken Kagaku Co., Ltd., Tokyo (*18*).

Proteolysis of Native Ovalbumin with S. griseus Proteinase—The enzymatic hydrolysis was performed in *M*/50 sodium phosphate buffer at pH 6.5 and at 25°C, with the use of lyophilized ovalbumin, which had been stored at 0° to 5°C for less than 3 weeks. The concentration of substrate in the solution was maintained to be 2.5 per cent, while that of enzyme 0.1 to 0.5 mg. per ml.

The rate of hydrolysis was estimated from the amount of amino compounds soluble in 2% perchloric acid (lower peptides and amino acids (*22*)) produced under the action of proteinase. The solution, after proteolysis of a given period, was added with five times volume of 2.5% perchloric acid, and the mixture was kept for one hour at room temperature. Preliminary experiments indicated a quantitative precipitation of the protein moiety without any further proceeding of hydrolysis under these conditions.

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* Abbreviation for amino acid residues is that suggested by Brand and Edsall. DNP is used for the abbreviation of 2, 4-dinitrophenyl.

An aliquot of the filtrate was used for the spectrophotometric determination of peptides (and amino acids)* produced, according to the 2, 4, 6-trinitrobenzene-1-sulfonic acid method of Satake *et al.* (*23*, *24*) and their amounts were expressed in mole, assuming the molar extinction coefficient of trinitrophenyl peptide in acid solution to be 1.05×10^4 (*23*).

Analysis of Perchloric Acid Soluble Fraction—Another portion of the above mentioned filtrate was made to be slightly alkaline (pH 8.2) with sodium bicarbonate, and was dinitrophenylated with 1-fluoro-2, 4-dinitrobenzene. The resulting DNP-derivatives, after being fractionated by ether- and ethylacetate-extractions, and dinitrophenol being removed by sublimation, were resolved by paper chromatography with the use of *n*-butanol saturated 5% ammonia and 1.5 *M* sodium phosphate buffer at pH 6, as the two solvent systems. The presence of perchloric acid did not cause any trouble in these procedures.

For quantitative determinations, the yellow spots on paper were eluted separately with water, and optical density at 360 mμ was determined for each extract (molar extinction coefficient of DNP-peptide being assumed to be 1.6×10^4). N-terminal and non-N-terminal amino acids analyses were achieved by hydrolysis of DNP-peptide in a sealed tube with 6 *N* hydrochloric acid at 110°C for 12 hours, fractionation with ether-extraction, and identification of both fractions, ether-soluble (DNP-amino acid from N-terminal) and insoluble (free amino acid from non-N-terminal), by paper chromatography. A mixture of *n*-butanol, acetic acid and water (4 : 1 : 2, v/v) and phenol saturated with water were used as the solvent systems for free amino acids.

Analysis of Protein Fraction—Paper electrophoretic analyses were performed on the whole enzymatic hydrolysate without any fractionation, while the N-terminal residue(s) of protein moiety after proteolysis, was analyzed on the precipitates derived with 2% perchloric acid.

Paper electrophoresis of protein was achieved on a paper strip, Toyo Roshi No. 51 A of 5×30 cm., impregnated with M/30 sodium phospoate buffer at pH 6.8 (*25*). After the two hours-electrophoresis under a potential gradient of 30 volt per cm., the protein (but not smaller peptides) on paper was stained with bromophenol blue reagent.

The precipitates derived from enzymatic hydrolysate with 2% perchloric acid as described above, were thoroughly washed with 2% perchloric acid, and the insoluble materials was dinitrophenylated. The method and the following procedures for hydrolysis of DNP-protein and for the fractionation and identification of the resulting α-DNP-amino acid, were the same as described in the preceding section.

RESULTS

Ovalbumin, in native state, produced scarcely small peptide fragments soluble into perchloric acid under the action of *S. griseus* proteinase (Pronase) at pH 6.5** and at 25°C, while the heat-denatured one was splitted into small fragments (about 60 per cent of the peptide linkages being hydrolysed (*26*~*28*)) very rapidly, indicated that the Pronase acted only on the denatured protein. Even from native ovalbumin, however, there were produced a minute amount of small fragments soluble in 2% perchloric acid, and their amounts were close to 2 moles per mole of ovalbumin (5×10^4g.) in every experiment. These results are shown in Fig. 1.

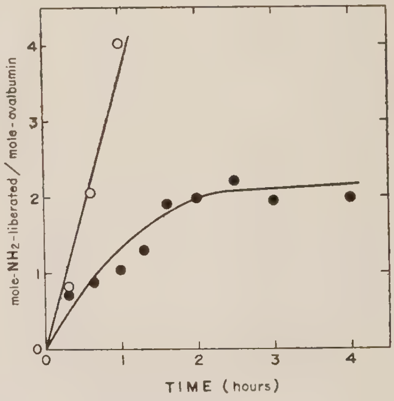

FIG. 1. Proteolysis of native and heat-denatured ovalbumins with *Streptomyces griseus* proteinase.

—●—: native ovalbumin,

—○—: heat-denatured ovalbumin.

protein : enzyme (250 : 1, w/w), pH 6.5, 25°C.

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* Usually, the amounts of free amino acids were negligible.

** The optimum pH of this proteinase was reported to be pH 8.

Such a minute amount of small fragments, although the amount being reproducible in every experiment using various concentration of proteinase (0.1 to 0.5 mg. per ml.), might be derived from denatured ovalbumin or other proteins contaminated in the preparation used. Paper electrophoretical patterns of protein moieties before and after the treatment with *S. griseus* proteinase, however seemed to exclude such a possibility. It was because that all ovalbumin was converted into another protein different in their electrophoretic mobilities after the splitting of about two moles of the small fragments. The results are shown in Fig. 2.

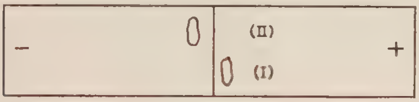
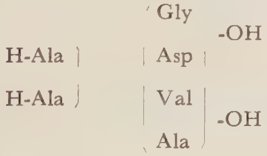


FIG. 2. Paper electrophoretic patterns of native and Pronase-modified ovalbumin.
(I): native ovalbumin
(II): Pronase-modified ovalbumin
Colored with bromophenol blue
Buffer: *M*/50 sodium phosphate, pH 6.8
Filter paper: Toyo-Roshi No. 51A
Condition: 30 volt per cm., 2 hours

Analyses on the N-terminal and amino acid composition of small fragments further verified that there occurred a specific limited proteolysis. Thus alanine was the sole N-terminal residues and only four kinds of amino acid, glycine, valine, aspartic acid and alanine, were found to be their constituents. The relative ratio of these amino acids was approximating to 1 : 1 : 1 : 3 in the whole acid hydrolysate and 1 : 1 : 1 : 1 in the non-N-terminal fraction after the terminal alanine being removed as the DNP-derivative.



$$\begin{array}{ll}\text{H-Ala} & \begin{pmatrix}\text{Gly}\\ \text{Asp}\end{pmatrix}\text{-OH}\\ \text{H-Ala} & \begin{pmatrix}\text{Val}\\ \text{Ala}\end{pmatrix}\text{-OH}\end{array}$$

After the liberation of the small peptide fragments, there appeared one kind of N-terminal (serine) in the Pronase-modified ovalbumin, which could be isolated as plate-like crystals, with the addition of ammonium sulfate at pH 4.5. The procedures are summarized in Scheme I.

SCHEME I.

Limited proteolysate of native ovalbumin at pH 6.5
↓ adjusted to pH 4.5 with 0.1 N H_2SO_4 (Pronase action was negligible)
↓ added with $(NH_4)_2SO_4$ to 0.5–0.6 saturation
↓ settled overnight at 4°C
Crude crystal
↓ centrifugation
Precipitation materials
↓ dissolved in the minimum amount of H_2O
↓ adjusted to pH 4.5 with 0.1 N H_2SO_4
↓ added with $(NH_4)_2SO_4$ to 0.5–0.6 saturation
↓ settled overnight at 4°C
Plate-like crystal

From the prolonged hydrolysate which had liberated 2.3 to 2.4 moles of small fragments, some other amino acids valine, glycine and alanine were identified as the additional N-terminals of the protein moieties, and valine, glycine and aspartic acid as the additional N-terminals of the small fragments. The total amounts of these additional N-terminals were less than one fourth of the main residues (serine in the protein moiety; alanine in the splitted small fragments) in both fractions.

Fig. 3 is a compositive paper chromatogram of dinitrophenylated small fragments and their constituents are summarized in Table I. There were observed a number of components, but the main ones were always S-2 (Ala-Ala) and S-1 (Ala-Gly-Val-Asp). The structure of tetrapeptide was confirmed to be Ala-Gly-Val-Asp by obtaining DNP-Ala-Gly and Val-Asp (as DNP-derivative) from the prolonged Pronase-hydrolysate (2.5 hours, 37°C, at pH 8.0 instead of pH 6.5) of DNP-Ala (Gly, Val, Asp) eluted from paper. Some amounts of DNP-Ala, DNP-Val and S-3 (Ala-

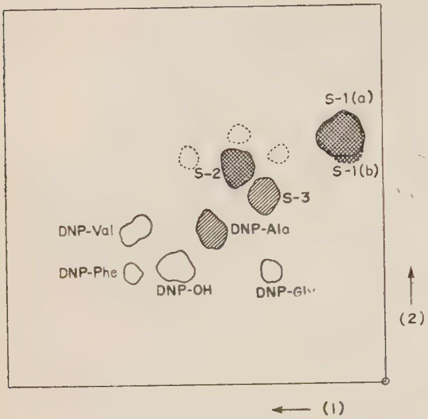


FIG. 3. Paper chromatogram of peptides (as DNP-derivatives) liberated from ovalbumin. Solvents: (1) *n*-butanol saturated with 5% ammonia, (2) 1.5 *M* phosphate buffer, pH 6.0 Filter paper: Toyo-Roshi, No. 51A

TABLE I

Identification of Small Fragments

No.[1]	N-terminal	non-N-terminal
S-1 (a)	Ala (trace Val)	Gly≈Val≈Asp
S-1 (b)	Ala≫Val	Asp>Val≈Gly
S-2	Ala	Ala
S-3	Ala	Gly

[1] See Fig. 3.

The amount of each amino acid was roughly estimated by densitometry of ninhydrin-colored paper chromatogram.

(a) obtained from 2/3 hours-hydrolysate

(b) obtained from 3.0 hours-hydrolysate

TABLE II

The Amounts of Peptide and N-Terminal Serine of Protein Moiety at Different Stages of Limited Proteolysis

protein: enzyme (125: 1, w/w), *M*/50 phosphate buffer, pH 6.5, 25°C

Proteolysis	1/3	2/3	1.0	2.0	3.0 (hr.)
Ala-Ala	0.42	0.80	0.96	0.96	0.90 moles[1]
Ala-Gly-Val-Asp	0.22	0.39	0.42	0.61	0.83
Val-Asp	±	±	+		
Asp	—	—	±		
Ala-Gly	0.03	0.09	0.18	0.26	0.27
Ala	0.02	0.25	0.29	0.36	0.36
Val	—	—	0.16	0.18	0.25
Gly	—	—	—	0.06	0.06
N-terminal Ser	2/1		1.0		3.0 (hr.)
	0.79		0.74		0.87 moles[1]

[1] moles per mole of ovalbumin

Gly) were observed only in the prolonged proteolysate of ovalbumin. The S-1 derived from such a proteolysate, although being mainly composed of the above mentioned tetrapeptide, was not homogenous. Thus the hydrolysate of extract of S-1 gave some amount of DNP-Val, together with DNP-Ala, while the amounts of glycine and valine from non-N-terminal were less than that of aspartic acid (see Table I). The results seem to suggest that S-1 would be contaminated with some amounts of DNP-Val-Asp. The coexistence of Ala-Gly (S-3) with Val-Asp will indicate slow hydrolysis of Ala-Gly-Val-Asp (S-1) under the action of Pronase.

These small fragments and the N-terminal serine in Pronase-modified ovalbumin were liberated with velocities as shown in Table II.

DISCUSSION

The results described above indicated that Pronase (*S. griseus* proteinase) catalyzed a limited proteolysis of native ovalbumin, giving another native protein, Ala-Ala and Ala-Gly-Val-Asp. The Pronase-modified protein could be isolated as plate-like crystls, migrated as slightly less acidic protein than the original one by paper electrophoresis (presumably due to the removal of one aspartyl residue) and possessed serine as the sole N-terminal residue.

As far as these facts are concerned, the transformation is very similar to the subtilisin (*B. subtilis* proteinase)-catalyzed conversion of ovalbumin into plakalbumin, which had been investigated in detail by Ottesen (*1*, *2*, *5*, *6*, *16*), although there have been no direct evidences indicating that the peptide fragments liberated under the action of subtilisin (Glu-Ala-Gly-Val-Asp-Ala-Ala)* and Pronase (Ala-Gly-Val-Asp, Ala-Ala) were derived from the same part of ovalbumin molecule. According to Ottessen, the subtilisin-catalyzed transformation was initiated by the rapid hydrolysis of alanyl-seryl linkages** in ovalbumin, and was followed with the hydrolysis of X-glutamyl and aspartyl-alanyl linkages. Thus there was observed formation of heptapeptide, Glu-Ala-Gly-Val-Asp-Ala-Ala, as an intermediate product.

$$\rightarrow\underbrace{(\text{Glu–Ala–Gly–Val–Asp}\overset{\downarrow}{–}\text{Ala–Ala–OH}\downarrow\text{H-Ser})}_{\text{plakalbumin}}$$

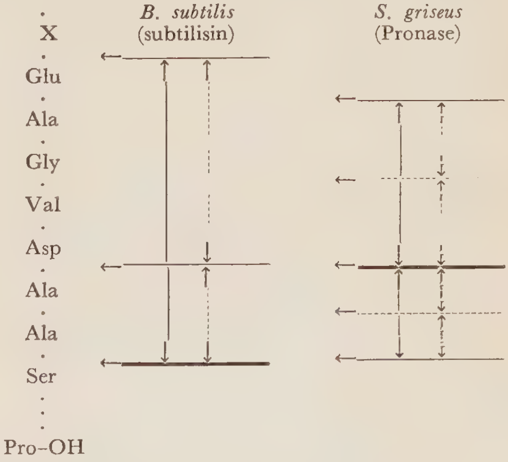
In case of Pronase-catalyzed transformation, Ala-Gly-Val-Asp and Ala-Ala were liberated without any accumulation of such an intermediate. The relative ratio of 1/2 observed in the velocities of Ala-Gly-Val-Asp- and Ala-Ala-formation (Table II) might permit some accumulation of Ala-Gly-Val-Asp-Ala-Ala in the partial hydrolysate, if it were for the fact that the Pronase-catalyzed transformation is initiated by the rapid hydrolysis of alanyl-seryl linkage.

From these speculations, it was presumed that the limited proteolysis would be initiated by the rapid hydrolysis of aspartyl-alanyl linkage as shown in Scheme II.

Scheme II

Limited Proteolysis of Ovalbumin

Proteinase from



As shown in Fig 3, there was observed a slow liberation of additional small fragments (Ala, Ala-Gly, Val-Asp), after the rapid liberation of Ala-Ala and Ala-Gly-Val-Asp. These products will be derived from the above mentioned two peptides, as the formation of free alanine was accompanied with the decrease in the accumulated Ala-Ala, and the formation of Ala-Gly with the liberation of comparable amount of Val-Asp. These results are not inconsistent with the substrate specificity of Pronase already elucidated both by Nomoto with the use of synthetic substrate (*30*) and by Satake on the denatured hemoglobin (*26*, *31*) and histone (*27*). On a further action of Pronase, there were also produced minute amounts of valine and aspartic acid. These free amino acids, however, might be derived from the protein moiety, instead of Val-Asp. It is because that their liberation was always accompanied with those of other amino acids, such as threonine, leucine, and phenylalanine.

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* In early papers (*5*, *6*), with the use of crude subtilisin, Ala–Gly–Val–Asp–Ala–Ala was reported to be the products.

** Such a selective hydrolysis of alanyl-seryl linkage in ovalbumin, is also presumed to occur under the action of carboxypeptidase untreated with diisopropylfluorophosphate (*29*)

Recently, Haruna (*13*) reported a quite different type of limited proteolysis of native ovalbumin. Thus a crystalline proteinase (Nagase) obtained from a strain of *B. subtilis* (*14*, *15*) catalyzed a transformation of ovalbumin into H-albumin, liberating three peptide fragments consisted of approximately twenty amino acid residues. It will be of interest that both proteinases, subtilisin and Pronase, obtained from quite different sources, catalyzed very similar type of limited proteolysis, while subtilisin and Nagase from bacteria of the same species did fairly different type. It should be emphasized here, that a partial hydrolysate of oxidized insulin B with Nagase possessed a a composition close to that derived with subtilisin which had been reported by Tuppy (*3*), while Pronase catalyzed the hydrolysis of oxidized insulin B with substrate specificity wider than that of Nagase.*

It has been well established by Okunuki *et al.* (*32*) that Nagase acts only on the unfolded polypeptide chain so strictly that the enzyme can be used for the selective assay of denatured protein. Subtilisin (*4*) and Pronase also possess such selectivity on the unfolded peptide chain. Their specificity, however, may differ from that of Nagase. From such differences among their steric specificity against unfolded peptide chain, different types of limited proteolysis of native ovalbumin might be derived.

SUMMARY

1. *Streptomyces griseus* proteinase (Pronase) catalyzed a limited proteolysis of native ovalbumin in to plakalbumin-like protein.

2. Two peptide fragments, Ala-Ala and Ala-Gly-Val-Asp were liberated. Relative velocities of the both liberation were approximately 2:1.

3. The Pronase-modified ovalbumin possessed one serine as the N-terminal and migrated as slightly less acidic protein than the original one by paper electrophoresis.

4. These results were discussed.

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The Journal of Biochemistry, Vol. 50, No. 2, 1961

Reactivity of Some Acetylenic Mercuric Compounds

I. Antifungal Activity and its Mode of Action

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(Received for publication, January, 23, 1961)

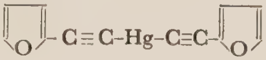
Two series of acetylenic mercuric compounds, represented by the general formulae $(R\text{-}C{\equiv}C)_2\text{-}Hg$ and $R\text{-}C{\equiv}C\text{-}Hg\text{-}R'$, were found to exhibit high antimicrobial activity against fungi and bacteria (*1*). The structures of these compounds are characteristic in that the two mercuric bonds are ionically less-dissociable and one or both of them are linked with ethynyl radical. These points are quite different from those mercuric compounds, such as phenylmercuriacetate and mercuric chloride, generally known as antimicrobial agents.

Present investigation was carried out to reveal the mode of action of these mercurials in comparison with that of known mercurials with special reference to the reactivity of these substances with sulfhydryl compounds or enzymes.

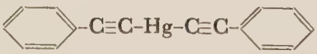
EXPERIMENTALS

Mercuric Compounds—Acetylenic mercuric componds* were kindly supplied by Dr. I. Iwai of this laboratory. Chemical structures of these compounds are as follows:

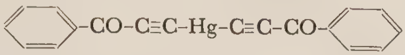
difurylethynyl mercury



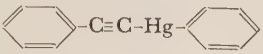
diphenethynyl mercury



dibenzoylethynyl mercury



phenylphenethnyl mercury



The other mercurials used as controls were of commercial origin.

Test Organism—A plant pathogen, *Cochliobolus miyabeanus* strain C-35, was used as test organism throughout these experiments. After 2 weeks of subculture at 27°C on a potato-sucrose agar slant, spores were harvested and suspended in distilled water. They were filtered through 3 sheets of gauze to remove other forms of cell, washed twice, and resuspended in 3 ml. of distilled water per slant.**

Sporostatic Test—An aliquot of the spore suspension was inoculated into a definite amount of culture medium supplemented with various amounts of a mercurial, then a small amount of the culture was subjected to drop-culture in a moisture chamber. After 4 hours of incubation at 27°C, the culture was examined microscopically to observe the rate of germination.

Medium used for these germination experiments was potato-sucrose medim unless otherwise specified. The degree of inhibition (*H*) of spore germination was calculated by the following equation:

$$H=(1-\frac{S}{S_0})\times 100 \text{ (per cent)},$$

in which S and S_0 represent percentages of germination in cultures with and without addition of a mercurial, respectively.

Preparation and Activity Mesurements of Enzymes—Crude urease was prepared from "urease powder" of jack bean (General Biochemical Inc.) according to the method of Kawerau (*3*). Ureolytic activity was determined in phosphate buffer (*M*/50, pH 7.0) as described by Sumner (*4*), although temperature and time of incubation were modified (25°C, 30 minutes) and further gum arabic was omitted. Conway's microdiffusion method was used for the determination of ammonia formed from urea used as a substrate.

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* The synthetic procedures of these compounds were reported elsewhere (*2*).

** All these procedures were performed within 15 minutes, since it was observed that some spores were able to sprout germ tubes in distilled water within about 40 minutes after being suspended in water.

Crude β-amylase was prepared from sweet potato according to the method of Balls (*5*) with a slight modification: one % calcium acetate was used instead of basic lead acetate. Socalled "crude paste" thus obtained was used as an enzyme preparation (specific activity being 137 Balls' units/mg. of protein). Enzyme reaction was carried out in an acetate buffer solution of pH 4.5 at 30°C and the amount of reducing sugar thus formed was determined by the method of Fehling-Lehman-Schoorl (*6*). Alcohol dehydrogenase was prepared according to the method of Racker (*7*), using fresh baker's yeast as a starting material. The purification of the enzyme was run until the first fractionation step using ammonium sulphate. The enzyme preparation thus obtained gave a specific activity of 22,200 Racker's units/mg. of protein. The activity of this enzyme was estimated by the method developed by the same author.

In each enzymatic experiment, the effect of mercurials on the activity was expressed in terms of per cent inhibition (H) calculated as follows:

$$H=(1-\frac{A}{A_0})\times 100 \text{ (per cent)},$$

in which A and A_0 represent relative activities of enzyme solutions with and without addition of a mercurial, respectively.

RESULTS

Sporostatic Activity of Mercurials—The inhibitory effects of a number of acetylenic mercuric compounds and other mercurials on *Cochliobolus miyabeanus* are illustrated graphically in Fig. 1.

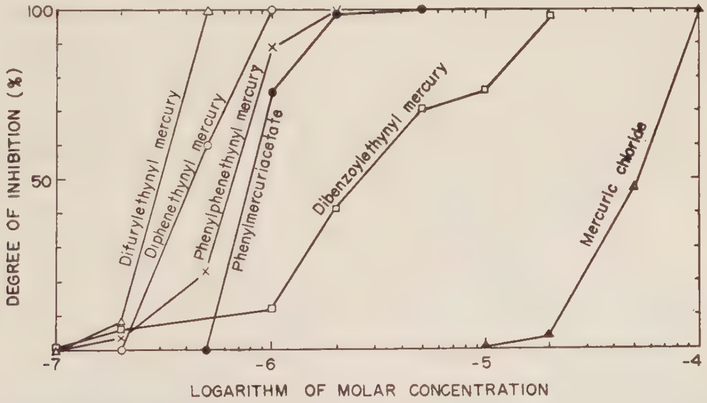

Fig. 1. Inhibitory effect of mercurials on spore germination of *Cochliobolus miyabeanus*.

These data showed that the most of acetylenic mercurials tested were as active as phenylmercuriacetate in their sporostatic activity. Of these mercurials diphenethynyl mercury and difurylethynyl mercury were the most active and their concentrations exhibiting half maximum inhibition were about 5×10^{-7} M.

Effect of Glutathione on Sporostatic Activity of Mercurials—Antifungal activity of known mercurials, such as phenylmercuriacetate and mercuric chloride, has been reported to be reversed by the addition of sulfhydryl compounds (*8*). Contrary to these easily dissociable mercurials, diphenethynyl mercury seems to be less-dissociable. The latter was therefore subjected to test whether or not the sporostatic activity might be affected by glutathione and EDTA,* taking phenylmercuriacetate and mercuric chloride as control substances. When diphenethynyl mercury and phenylmercuriacetate were added to spore suspension simultaneously with glutathione, a profound decrease in the inhibitory activity was observed in both cases (Figs. 2 and 3). Thus, as the concentration of glutathione increased, sporostatic activity changed inversely. EDTA exhibited contrarily no appreciable effect on the sporostatic activity of these two mercurials, whereas it caused the loss of sporostatic activity of

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* EDTA has been known to show some alleviatory effect on copper toxicity against fungal spore (*9*).

TABLE I

Effect of EDTA on Sporostatic Activity of Mercurials

Test organism: *Cochliobolus miyabeanus.*

Mercurial solution mixed with basal medium (see Fig. 2) was incubated for 20 minutes at 27°C in absence or presence of EDTA, then sporostatic activity of such solution was estimated.

Mercurial	In absence of EDTA				In presence of EDTA[1]			
Concentration (*M*)	Total number of spore examined	Number of spore germinated	Degree of germination (%)	Degree of inhibition (%)	Total number of spore examined	Number of spore germinated	Degree of germination (%)	Degree of inhibition (%)
Diphenethynyl mercury								
0	140	140	100	(0)	103	103	100	(0)
2×10^{-7}	139	107	77	23	110	78	71	29
5×10^{-7}	100	6	6	94	112	0	0	100
10^{-6}	105	0	0	100	126	0	0	100
Phenylmercuriacetate								
0	93	89	96	(0)	74	71	96	(0)
3×10^{-7}	117	98	84	12	124	111	90	7
6×10^{-7}	94	35	37	61	113	32	28	71
8×10^{-7}	81	0	0	100	109	3	3	97
Mercuric chloride								
0	124	118	95	(0)	148	140	95	(0)
5×10^{-5}	137	0	0	100	155	150	97	−2
10^{-4}	99	0	0	100	136	124	91	4
5×10^{-4}	95	0	0	100	190	79	42	56

1) Concentration of EDTA: 10^{-4} *M* for diphenethynyl mercury and phenylmercuriacetate, 2×10^{-3} *M* for mercuric chloride.

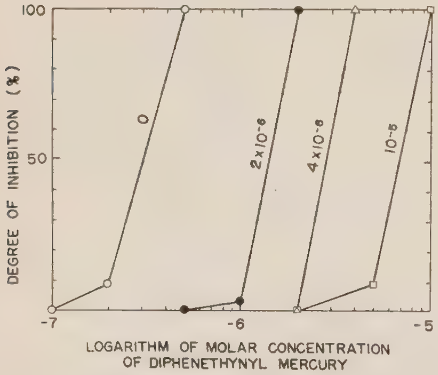

FIG. 2. Effect of glutathione on sporostatic activity of diphenethynyl mercury.

Test organism: *Cochliobolus miyabeanus.*

To basal media (1 per cent glucose, 0.05% urea, and *M*/50 phosphate buffer; pH 6.0) containing various amounts of this mercurial, glutathione was added immediately before inoculation of spores. Figures indicated are molar concentrations of glutathione.

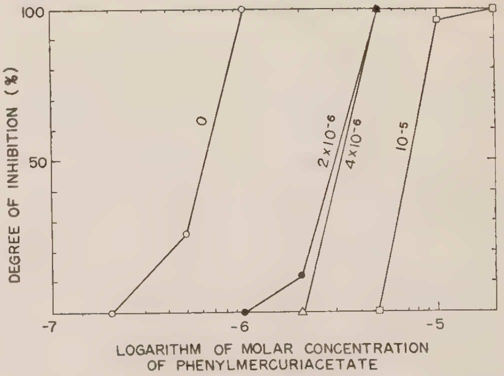

FIG. 3. Effect of glutathione on sporostatic activity of phenylmercuriacetate.

Test organism: *Cochliobolus miyabeanus.*

Experimental procedure was followed as explained in Fig. 2. Figures indicated are molar concentrations of glutathione.

mercuric chloride as shown in Table I.

Inhibitory Effects of Mercurials on Sulfhydryl Enzymes—Since glutathione showed antagonistic effect on the sporostatic activity of acetylenic mercuric compounds, it is highly probable that these mercuric compounds interact with sulfhydryl protein in the cell as is the case in other mercurials conventionally used as antimicrobial agents. Therefore, the effect of these acetylenic mercuric compounds on sulfhydryl enzymes, such as urease, β-amylase, and alcohol dehydrogenase was tested.

In Fig. 4 are shown the dosage-response curves concerning the inhibitory effect of various mercurials upon the activity or crude urease. Of these mercuric compounds, it was revealed that mercuric chloride and phenylmercuriacetate were the most effective (complete inhibition occurred at about 10^{-6} *M*), phenylphenethynyl mercury and dibenzoylethynyl mercury were less active than the formers (complete inhibition occurred at about 10^{-5} *M*), and diphenethynyl mercury and difurylethynyl mercury were effective only slightly at each concentration of nearly maximum solubility of these compounds.

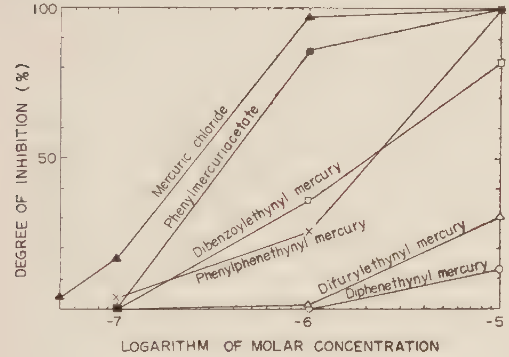

Fig. 4. Inhibitory effect of mercurials on activity of jack bean urease.

Urease (0.06 units/ml.) dissolved in *M*/50 phosphate buffer (pH 7.0) was incubated for 30 minutes at 25°C in absence or presence of graded concentrations of each mercurial, then activity of enzyme solution thus treated was estimated. Reaction system for assay of enzymatic activity: 1 ml. of enzyme solution and 2 ml. of buffer containing 0.3% urea.

In the next experiment, diphenethynyl mercury, one of these acetylenic mercuric compounds, was tested for its inhibitory

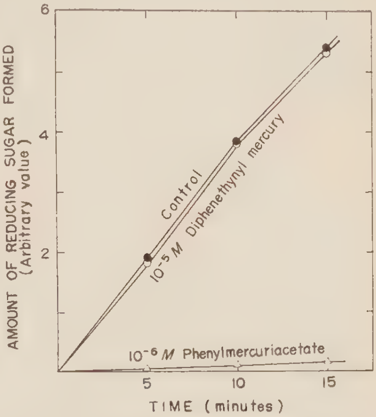

Fig. 5. Effect of diphenethynyl mercury on activity of sweet potato β-amylase.

Experimental procedure was followed as explained in Fig. 4 except that *M*/50 acetate buffer (pH 4.5) was used in pretreatment. Concentration of β-amylase: 4.8 units/ml. Reaction system for assay of enzymatic activity: 1 ml. enzyme solution, 7 ml. of 3% soluble starch, and 2 ml. of *M*/10 acetate buffer (pH 4.5).

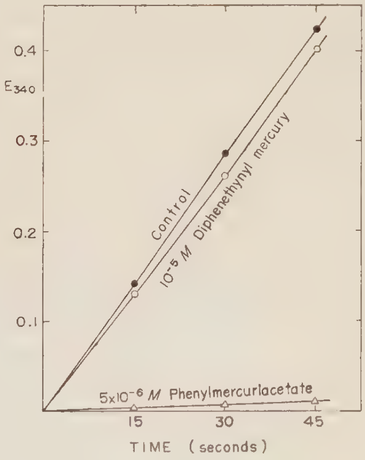

Fig. 6. Effect of diphenethynyl mercury on activity of yeast alcohol dehydrogenase.

Experimental procedure was followed as explained in Fig. 4, except that pretreatment was carried out in *M*/50 phosphate buffer (pH 7.6) containing 0.2% glycine for 10 minutes. Concentration of alcohol dehydrogenase: 1740 units/ml. For assay of enzymatic activity, neither gelatin nor serum albumin was used.

activity on the other sulfhydryl enzymes, β-amylase and alcohol dehydogenase, in comparison with phenylmercuriacetate. As shown in Figs. 5 and 6, no significant effect was observed at a level, at which phenylmercuriacetate exhibited complete inhibitory activity on these enzymes.

DISCUSSION

Mercuric compounds used in the test for sporostatic activity on a plant pathogen, *Cochiliobolus miyabeanus*, may be classified roughly into three groups. One is characterized in their molecules by two ionically less-dissociable bonds (difurylethynyl mercury, diphenethynyl mercury, phenylphenethynyl mercury, and dibenzoylethynyl mercury) and the two others are characterized by one or two easily dissociable bonds (phenylmercuriacetate and mercuric chloride). As the result of the present experiments, mercurials of the first group were shown to be as active as the latter two groups. It was also found that sulfhydryl compound, such as glutathione, seemed to antagonize the sporostatic activity of the first group substances as well as that of the other groups. Only difference observed among these groups was the effect of EDTA on the sporostatic activity. The activity of mercuric chloride, which has two easily dissociable bonds, was found to be antagonized by EDTA, while that of the other mercurials tested was unaffected. Moreover it was already pointed out in the previous paper (*1*) that diphenethynyl mercury (endowed with two less-dissociable bonds) showed quite a similar antimicrobial spectrum as compared with phenylmercuriacetate (endowed with one easily dissociable bond).

From these results one is tempted to consider that these mercurials of the first group react with some sulfhydryl enzymes which are generally accepted to be the site of action of second and third groups of mercurials.

The results of the test on this point, however, were of quite unexpected. In contrast to the fact that the second and third group mercurials were highly effective on urease, β-amylase, and alcohol dehydrogenase, the first group mercurials were shown to be ineffective or slightly effective, if any, on these enzymes. Although the varieties of sulfhydryl enzymes tested were only limited, the results obtained were thus entirely remained unsolved. The fact that the sporostatic activity of diphenethynyl mercury was antagonized by glutathione seemed to be worthy of notice. This fact suggests the occurrence of chemical interaction between glutathione and diphenethynyl mercury which has no easily dissociable bonds in its molecule. This point will be dealt in the forthcoming paper which concerns with the mode of action of such less-dissociable acetylenic mercuric compounds.

SUMMARY

Acetylenic mercuric compounds of general formulae $(R{-}C{\equiv}C)_2Hg$ and $R{-}C{\equiv}C{-}Hg{-}R'$ were found to be highly effective in inhibiting spore germination of a plant pathogenic fungus, *Cochliobolus miyabeanus*. This antifungal activity was shown to be reversed in the presence of glutathione.

This fact suggests that those mercurials may be active against sulfhydryl enzymes as is the case in the other known organomercuric compounds. It was, however, shown that some of those acetylenic mercuric compounds were almost ineffective against β-amylase of sweet potato and alcohol dehydrogenase of baker's yeast and slightly effective against jack bean urease. The ineffectivity of such mercurials against sulfhydryl enzymes is discussed.

The author wishes to express his deep gratitude to Prof. T. Yanagita of Chiba University and Prof. A. Takamiya of University of Tokyo for their kind guidance and encouragement throughout this work. Thanks are due to Mr. M. Matsui, Director, and Dr. Y. Miura of this laboratory for their kind encouragement. The author greatly appreciates the kindness of Dr. I. Iwai of this laboratory for his supply of acetylenic mercurials.

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The Journal of Biochemistry, Vol. 50, No. 2, 1961

Reactivity of Some Acetylenic Mercuric Compounds

II. Spectrophotometric Study of Reactivity of Diphenethynyl Mercury

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(Received for publication, January 23, 1961)

Some aspects on the biochemical reactivity of several acetylenic mercuric compounds which were active against fungal spore germination were described in the preceding paper. Of these mercurials used, diphenethynyl mercury, which was thought to be less-dissociable ionically, did not inhibit significantly the activity of three sulfhydryl enzymes, such as urease, β-amylase, and alcohol dehydrogenase, although it seemed to react with glutathione.

Comparing with sulfhydryl groups in molecules of most of low-molecular-weight compounds, those in protein molecules were frequently found to be less reactive with various sulfhydryl reagents, although some of them were as reactive as those of low-molecular-weight compounds. Such less reactive sulfhydryl groups in protein molecules were designated as sluggish groups to differentiate from the other reactive groups (*1*).

According to the data obtained by Hellerman (*2*), urease protein molecule contains also such sluggish sulfhydryl groups and one half of the total number of sulfhdryl groups in the molecule are occupied by these sluggish groups, which are considered to be directly involved in the ureolytic activity of this enzyme.

It may be probable, therefore, to consider that diphenethynyl mercury is not able to react with sluggish sulfhydryl groups of urease protein resulting in only a slight inhibition of the ureolytic activity, reserving the possibility that it may interact with reactive sulfhydryl groups of this enzyme protein.

On examining the validity of such considerations, the interaction of diphenethynyl mercury with sulfhydryl groups was traced spectrophotometrically, since it was found that this mercurial showed an intensive absorption spectrum in ultraviolet region, which was affected characteristically by the reaction with sulfhydryl compounds.

In the first part of this paper are described spectrophotometric investigations of the interaction of diphenethynyl mercury with glutathione and in the second part with urease protein.

MATERIALS AND METHODS

Materials—Diphenethynyl mercury and phenylacetylene were kindly supplied by Dr. I. Iwai of this laboratory. Freshly recrystallized or redistilled samples of these substances were used throughout these experiments. The other materials used were of commercial origin.

Preparation and Activity Measurement of Crystalline Urease—Crystalline urease was prepared from jack bean meal obtained from Sigma Company. The crystallization of urease from acetone-citrate buffer mixture was carried out according to the methods of Sumner (*3*) and Dounce (*4*), and the crystals thus obtained were freeze-dried and stored.

The determination of activity of this enzyme was performed under the same conditions as described in the preceding paper except that enzyme reaction was carried out for 3.5 minutes in a buffer supplemented with glycine. The protein content of urease solution was determined by micro-Kjeldahl procedure using the protein factor of 6.3.

Spectrophotometry—For the analysis of ultraviolet region, Beckman's ratio-recording spectrophotometer model DK-2 was used. For infrared analysis, Perkin-Elmer recording spectrophotometer model 21 was used.

RESULTS AND DISCUSSION

Part. I. Reaction of Diphenethynyl Mercury

with Glutathione

Over-all Reaction—In 30–35% (v/v) aqueous ethanol, diphenethynyl mercury shows a characteristic absorption spectrum in ultraviolet region characterized by two maxima at 254 and 264.5 mμ as illustrated in Fig. 1 by a continuous line. Incubation of diphenethynyl mercury with glutathione for 2 hours at 25°C at the molecular ratios of 1 : 1 (broken line) and 1 : 2 (dotted line) resulted in the alteration of the spectral pattern. It is thus apparent that the reaction of diphenethynyl mercury with glutathione causes an extensive decrease in absorbancy in the region of longer wave length than 247 mμ and definite increase in the region shorter than 247 mμ, and that such change is dependent upon the molar ratio of the reactants.

As can be considered from the ultraviolet absorption spectrum of phenylacetylene shown in Fig. 2-a (curve B), the spectral change appeared in the region of shorter wave length may be attributable to the formation of phenylacetylene in the reaction system. This view was further ascertained by an infrared spectrophotometrical study. As shown in Fig. 3-a, an infrared absorption spectrum of the carbon tetrachloride extract of the reacted solution of diphenethynyl mercury and glutathione, showed an absorption band

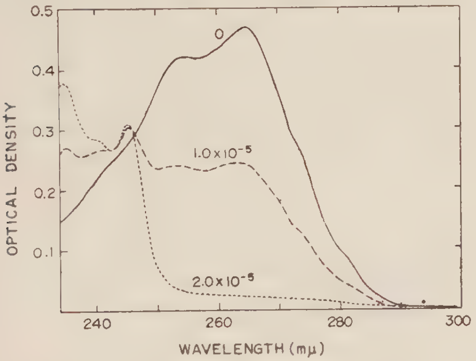


Fig. 1. Change in absorption spectrum of diphenethynyl mercury caused by addition of various concentrations of glutathione.

Reaction was carried out in 30% (v/v) aqueous ethanol for 2 hours at 25°C. Initial concentration of diphenethynyl mercury was $1.0 \times 10^{-5} M$. Figures indicated are molar concentrations of glutathione.

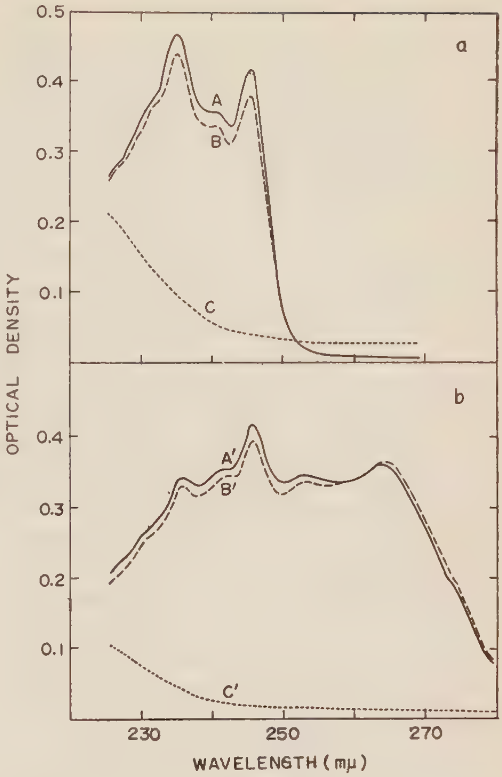


Fig. 2. Change in absorption spectrum of diphenethynyl mercury caused by addition of glutathione, demonstrating stoichiometric relationship of these reactants.

Spectrum was measured at end of reaction which was carried out in $M/50$ phosphate buffer (pH 7.0) cotaining 35% (v/v) ethanol for 2 hours at 25°C.

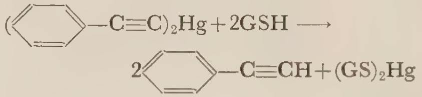
a. Molar ratio of glutathione to diphenethynyl mercury was 2.
   Spectrum A: Initial composition, $1.5 \times 10^{-5} M$ diphenethynyl mercury plus $3.0 \times 10^{-5} M$ glutathione. Reference solution, $1.5 \times 10^{-5} M$ diglutathionyl mercury.
   Spectrum B: $3.0 \times 10^{-5} M$ phenylacetylene against solvent.
   Spectrum C: $1.5 \times 10^{-5} M$ diglutathionyl mercury against solvent.
b. Molar ratio of glutathione to diphenethynyl mercury was 1.
   Spectrum A′: Initial composition, $1.5 \times 10^{-5} M$ diphenethynyl mercury plus $1.5 \times 10^{-5} M$ glutathione. Reference solution, $0.75 \times 10^{-5} M$ diglutathionyl mercury.
   Spectrum B′: Mixture of $0.75 \times 10^{-5} M$ diphenethynyl mercury and $1.5 \times 10^{-5} M$ phenylacetylene against solvent.
   Spectrum C′: $0.75 \times 10^{-5} M$ diglutathionyl mercury against solvent.

at 3,300 cm⁻¹ characteristic for stretching absorption of ethynyl radical ($-C{\equiv}CH$), whereas such an absorption band was not detected in infrared spectra of glutathione (b) and diphenethynyl mercury (c).

Further studies on the isolation of another product of this reaction were carried out as follows:

Four hundred and four miligrams of diphenethynyl mercury and 460 mg. of glutathione were dissolved in aqueous ethanol, molar ratio of glutathione to diphenethynyl mercury being 1.49. The mixture was left standing for 2 hours at 30°C in the dark. Then the resultant precipitate was collected, washed with ethanol and dissolved in a small volume of water. The solution was added again into aqueous ethanol supplemented with 10 mg. diphenethynyl mercury. After an incubation period of 2 hours, ethanol was poured into this mixture excessively, and the resultant precipitate collected and washed several times with ethanol, and finally dried *in vacuo*. Analytical values (per cent) obtained for this substance were found to be in good correspondence with those of diglutathionyl mercury as follows: Found: C, 29.21; H, 4.34; Hg, 25.00. Calculated for diglutathioyl mercury: C, 29.54; H, 3.97; Hg, 24.67.

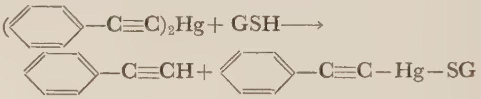
From the results obtained from preceding experiments, it was found that the reaction of diphenethynyl mercury with glutathione resulted in the formation of phenylacetylene and diglutathionyl mercury as final products. Therefore the over-all reaction may be formulated as follows:



(1)

in which GSH and $(GS)_2Hg$ denote glutathione and diglutathionyl mercury, respectively: these notations will be followed in later formulae.

When the molar ratio of glutathione to diphenethynyl mercury (denoted as ratio *r*) is less than 2, however, there are alternative possibilities that the reaction proceeds according to formula (2) or reactions represented by formulae (1) and (2) proceed in parallel.



(2)

To clarify this point, some stoichometric investigations were carried out. When the absorption spectrum of a mixture containing 1.5×10^{-5} *M* diphenethynyl mercury and 3.0×10^{-5} *M* glutathione ($r=2$) was taken after the reaction completed against a reference solution of 1.5×10^{-5} *M* diglutathionyl mercury, a

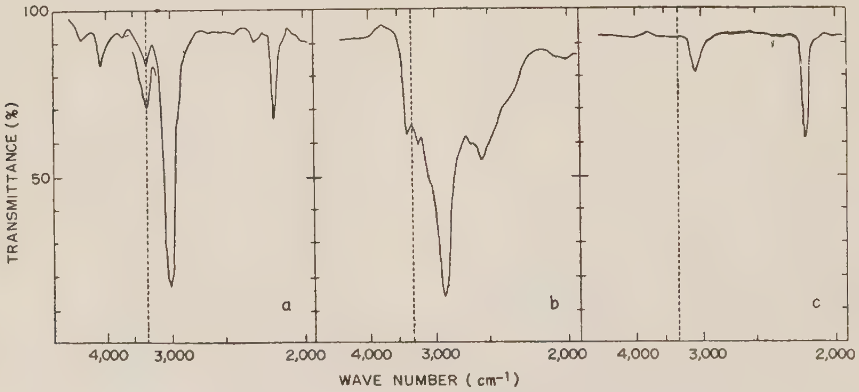


Fig. 3. Detection of ethynyl radical by infrared spectrophotometry in reaction mixture of diphenethynyl mercury and glutathione.

a. CCl_4 extract of reaction mixture of diphenethynyl mercury and glutathione in 30% (v/v) aqueous ethanol (in CCl_4).

b. Glutathione (in nujol).

c. Diphenethynyl mercury (in CCl_4).

spectrum of curve A in Fig. 2-a was obtained. If the over-all reaction proceeded according to formula (1), this spectrum should coincide with that of $3.0\times10^{-5}\,M$ phenylacetylene (curve B). As shown in this figure both spectra show good coincidence with each other.

Similar experiment was then carried out taking the initial concentrations of diphenethynyl mercury and glutathione to be equimolar ($1.5\times10^{-5}\,M$), with reference solution of $0.75\times10^{-5}\,M$ diglutathionyl mercury. Thus the reacted solution gave a spectrum of curve A′ in Fig. 2-b. If the over-all reaction proceeded according also to formula (1), one half portion of diphenethynyl mercury should remain unreacted and corresponding moles ($1.5\times10^{-5}\,M$ in concentration) of phenylacetylene should be produced. This consideration was proved to be valid, since the spectrum of curve A′ was found to be in fairly good correspondence with a spectrum of a mixture containing $0.75\times10^{-5}\,M$ diphenethynyl mercury and $1.5\times10^{-5}\,M$ phenylacetylene (curve B′).

In the latter case, when r was fixed to be unity, another reaction product was isolated from the reacted solution as follows:

To 10 ml. of 80% (v/v) aqueous ethanol solution of 21.8 mg. of diphenethynyl mercury, 0.8 ml. of aqueous solution of 15.6 mg. of glutathione ($r=0.94$) was added dropwise. White precipitant formed after 3 hours at 30°C in the dark was collected, washed with ethanol repeatedly, and finally dried *in vacuo.*

Although chemical analysis of this reaction product was not performed because of its small yield, this substance was assumed to be diglutathionyl mercury by the following reasons. 1. ultra- violet spectrum was identical with digluta- thionyl mercury and 2. no sign of the formation of phenylacetylene was detected when a sufficient amount of glutathione was added to its solution.

These observations mentioned above led us to the conclusion that 1 mole of diphenethynyl mercury gave finally 1 mole of diglutathionyl mercury and 2 moles of phenylacetylene by the reaction with 2 moles of glutathione, independent on the initial molar ratio of these two reactants. This suggests that even if a hypothetic intermediate,

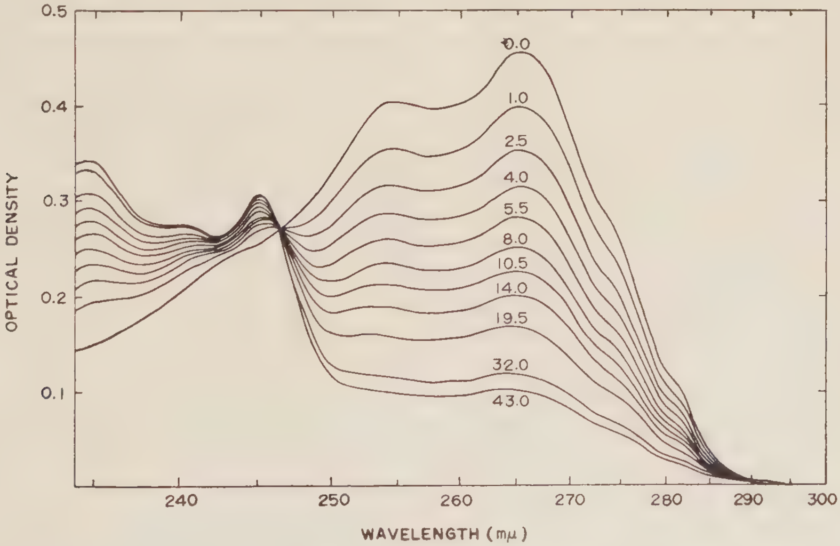


Fig. 4. Time course of change in absorption spectrum of reaction system composed of $1.0\times10^{-5}\,M$ diphenethynyl mercury and $3.0\times10^{-5}\,M$ glutathione in 70% aqueous ethanol (v/v).

Figures indicated are time (in minutes) after time of mixture of two reactants. In each measurement of spectrum, it took about 1.2 minutes.

phenethynyl-glutathionyl mercury ($C_6H_5-C\equiv C-Hg-SG$), might be formed, it would further react with glutathione resulting in the formation of the final product, diglutathionyl mercury.

Kinetics of the Reaction—Although over-all reaction between diphenethynyl mercury and glutathione was revealed to be 1 : 2 reaction on molar basis, the mechanism of reaction has not yet been elucidated. To clarify this point, the reaction was analysed kinetically using data obtained by similar spectrophotometric experiments.

As shown in Fig. 4, the time course of the change in absorption spectrum could be traced on this reaction system.* When change in absorbancy at 264.5 mμ of this reaction system was traced, one could obtain a curve showing the apparent decrease in concentration of diphenethynyl mercury as time proceeded (Fig. 5-a). In this case, however, it was found to be necessary to give a correction to these data in order to show true concentration of this mercurial, since glutathione, diglutathionyl mercury, and phenylacetylene exhibit slight but definite light absorption at this wave length. Therefore, calculation for this correction was carried out according to the following equation.**

$$E_t=\alpha_t+\beta+\sigma\frac{\alpha-\alpha_t}{\alpha}^{***} \qquad (3)$$

in which each notation represents as follows:

E_t: $E_{264.5}$ of reaction system as a whole at time t,

σ: net $E_{264.5}$ of final products ($E_{264.5}$ of remaining reactants is not included),

α and α_t: $E_{264.5}$ of diphenethynyl mercury at time zero and time t, respectively, and

β: $E_{264.5}$ of glutathione.

Equation (3) will then be changed to the equation:

$$\frac{\alpha}{\alpha_t}=\frac{\alpha-\sigma}{E_t-\sigma-\beta} \qquad (4)$$

In these experiments, concentrations of glutathione used varied between $5.0\times10^{-4}\,M$ and $4.0\times10^{-3}\,M$ and that of diphenethynyl mercury was fixed to be $10^{-5}\,M$. Thus, the concentrations of glutathione were always much more excessive than that of diphenethynyl mercury. Therefore the concentration of the former may be assumed to be nearly constant throughout the reaction.

Let us, then, denote diphenethynyl mercury molecule by A, its initial concentration by a and the concentration at a given time by a_t.

In Fig. 5-b, the logarithm of α/α_t is plotted against time. As α/α_t in this case corresponds to a/a_t, the linearity of the curve indicates that the decrease of diphenethynyl mercury proceeds in the first order reaction.

In Table I are listed time values of half decay, τ, of diphenethynyl mercury in the presence of various concentrations of glutathione. Each value of τ was determined by the same plotting as shown in Fig. 5-b. If the rate limiting process involves glutathione as one reactant, then

$$ln\frac{a}{a_t}=kb^{r}t\ (r=1 \text{ or } 2) \qquad (5)$$

or $$\tau=\frac{ln\ 2}{kb^{r}} \qquad (6)$$

where glutathione and its concentration are denoted by B and b, and k is a velocity constant.

Namely, the time of half decay τ should be inversely proportional to the concentration (or its square) of glutathione. As seen in Table I, data clearly show that this is not the case: τ seems to increase only slightly as the concentration of glutathione decreases widely.

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* The reaction demonstrated in this figure was carried out in 70% (v/v) aqueous ethanol. In order to keep the experimental conditions equal to the other trials, it was desirable to carry out the reaction in 35% (v/v) instead of 70% aqueous ethanol. In such a solution, however, the rate of reaction was so high that it was actually impossible to demonstrate the time course of spectral change over a wide range of wavelength.

** In this calculation, the change in absorbancy of gluthathione was ignored, since it was negligible.

*** When intial concentrations of diphenethynyl mercury and glutathione, for example, were $10^{-5}\,M$ and $10^{-3}\,M$, respectively, the value σ and β were observed to be 0.02 and 0.026, respectively.

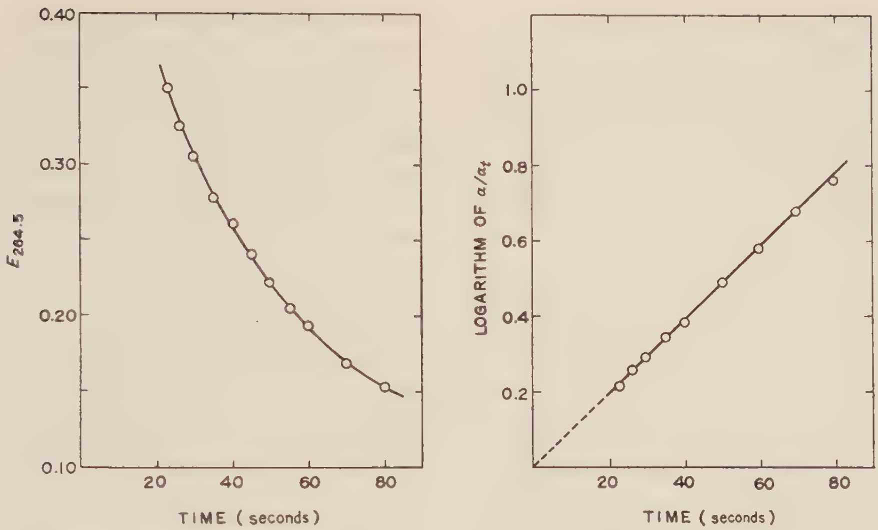

FIG. 5. Time course of change in absorbancy at 264.5 mμ of reaction system of diphenethynyl mercury and glutathione.

Initial concentrations of diphenethynyl mercury and glutathione were 1.0×10^{-5} *M* and 2.0×10^{-3} *M*, respectively. Reaction was carried out in *M*/10 acetate buffer (pH 5.3), containing 35% (v/v) aqueous ethanol at 27.5°C.

On definitions of α and α_t, see text. α/α_t was calculated according to equation (*4*).

TABLE I

Rate of Reaction of Diphenethynyl Mercury (1.0×10^{-5} M) with Various Amounts of Glutathione

Reactions were carried out in acetate buffer (*M*/10, pH 5.3) containing 35% (v/v) aqueous ethanol at 27.5°C.

Concentration of glutathione (*M*)	Time of half decay (in seconds)				
	Empirical value				Theoretical value[1]
	Exp. I	Exp. II	Exp. III	Mean	
4.0×10^{-3}	26.4	26.4	26.6	26.5±0.1	26.50
2.0×10^{-3}	27.7	34.1	30.7	30.7±3.4	28.77
1.0×10^{-3}	34.1	35.0	39.1	36.1±3.0	33.31
5.0×10^{-4}	40.8	36.0	41.8	39.5±3.5	42.40

1) Obtained by calculation according to equation (12) or (15), in which K or K' was assumed to be 3.75×10^{-4} and r to be 1.

Therefore, it may be pertinent to postulate alternative schemes as follows:

Scheme I

Let us denote 'the active molecule' of diphenethynyl mercury by A※ and its concentration by a', with an assumption that it has the same ultraviolet spectrum as that of A. The successive processes are then expressed by the followings:

$$A \underset{k_2}{\overset{k_1}{\rightleftharpoons}} A\text{※} \qquad (7)$$

$$A※+rB\xrightarrow{k_3}\sum Pi \qquad (8)$$

where k_1, k_2 and k_3 are rate constants in each process and Pi represents the reaction product.

On applying the steady state treatment on A※, then

$$k_1\ (a_t-a')=(k_2+k_3b^r)a' \qquad (9)$$

$$-\frac{d(a_t)}{dt}=k_3b^ra' \qquad (10)$$

hence

$$ln\ \frac{a}{a_t}=\frac{k_1k_3b^r}{k_1+k_2+k_3b^r}\ t \qquad (11)$$

or

$$\tau=\frac{ln\ 2}{k_1}(1+\frac{K}{b^r}) \qquad (12)$$

where K represents $(k_1+k_2)/k_3$.

If K is much smaller than b^r, τ may be constant at various concentrations of glutathione. On the other hand, if K is not so much small but not greater than the lowest concentration (or its square) of glutathione used, the values of τ may be obtained by calculation as shown in Table I. These values are, indeed, in good correspondence with those obtained empirically.

Thus it may be probable to consider that the foreward process of the initial reaction (equation 7) in this presumptive reaction scheme is one of the possible rate-limiting processes.

Scheme II

In this scheme, the formation of an intermediate complex of diphenethynyl mercury with glutathione is posturated. When the complex is denoted by ArB and its concentration by c, the reaction processes are then expressed by the following equations:

$$A+rB\underset{k'_2}{\overset{k'_1}{\rightleftharpoons}}ArB \qquad (13)$$

$$ArB\xrightarrow{k'_3}\sum Pi \qquad (14)$$

where k'_1, k'_2 and k'_3 are rate constants.

On applying the steady state treatment on ArB, calculations were likewise carried out. Finally then the following equation was obtained.

$$\tau=\frac{ln\ 2}{k'_3}(1+\frac{K'}{b^r}) \qquad (15)$$

where K' represents $(k'_2+k'_3)/k'_1$.

It is thus clear that equation (15) is the equal form to equation (12). In scheme II, therefore, the foreward process represented by equation (14) is the rate limiting reaction.

From these kinetic considerations, scheme I and II may be posturated as possible reaction mechanisms, although neither intermediate active form of diphenethynyl mercury nor intermediate diphenethynyl mercury-glutathione complex was determined to be present.

Part II. The Reaction of Diphenethynyl Mercury with Crystalline Urease

Ultracentrifugation Experiments—A solution of crystalline urease (2.85 mg./ml., 33,800 units/g. of protein) dissolved in phosphate buffer (*M*/40, pH 7.0) was subjected to measurement of sedimentation constant, using Spinco ultracentrifuge model E (full rotor speed was 59,780 r.p.m. and average temperature was 21.3°C). This urease preparation gave $S_{20,w}$ of 19.3 as a main peak, which was quite similar to the values of 18.6~19.9 obtained by Sumner and coworkers (*5*) for their urease sample. When 0.1 ml. of diphenethynyl mercury solution [2.2×10^{-4} *M* in 10% (v/v) ethanol] was mixed with 1 ml. of this urease solution and kept for 30 minutes at room temperature, ultracentrifugal characteristics changed remarkably. Thus, precipitates were formed readily and any sedimentation patten could not be observed.

After disturbing the sedimented centrifugate by stirring, 0.1 ml. of 1.2×10^{-2} *M* glutathione solution was added to it and kept for a short period, then ultracentrifugation was carried out again. Such a treatment resulted in the reappearance of a clear sedimentation pattern characterized by $S_{20,w}$ to be 18.7. It was thus concluded that diphenethynyl mercury might cause the aggregation of urease protein and this phenomenon was reversed by the addition of glutathione.

Ultraviolet Spectrum of Reaction Mixture—When diphenethynyl mercury was added into urease solution, apparent change in the spectrum of the mercurial was observed and the spectral patterns (Fig. 6) obtained after certain time periods were found to be similar to those obtained by the reaction of the mercurial with glutathione (Fig. 4). This suggests that the reaction of diphenethynyl mercury with urease is similar to the reaction discussed above.

In the kinetic analysis of this spectral change, some correction should have been made, since the basal line of the spectrum rose slightly as time proceeded. It was probably due to the appearance of a slight turbidity. Thus, extinction value, $E_{264\cdot5}-E_{295}$, was taken in determining the time course of this reaction.

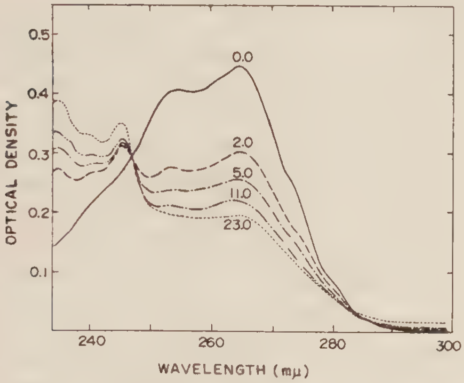

Fig. 6. Time course of change in absorption spectrum of diphenethynyl mercury caused by addition of urease. Reaction system: 0.93×10^{-5} *M* diphenethynyl mercury and 0.168 g. (in dry weight) per liter urease (15,200 units/g. of protein) in *M*/50 phosphate buffer (pH 7.0) containing 30% (v/v) aqueous ethanol. Control system containing no mercurial was used as a reference solution.

Figures indicated are time (in minutes) after time of mixture of two reactants.

Reaction of Purified Urease with Diphenethynyl Mercury—Since the reaction of diphenethynyl mercury with urease protein was found to be able to trace photometrically, the course of the reaction together with the change in the ureolytic activity of purified urease was observed to know how the reaction of urease with diphenethynyl mercury might affect the ureolytic activity of this enzyme.

A solution of crystalline urease (82,400 units/g. of protein) was mixed with a solution of diphenethynyl mercury, the amount of which was chosen to be equivalent to that of sulfhydryl groups contained in the urease sample employed, taking the sulfhydryl value of 44 per mole of urease protein (*2*, *5*), and the reaction was followed at 25°C.

In this experiment glycine was also added to the reaction mixture to eliminate a trace amount of copper ion, which is known to be a potent inhibitor on this enzyme activity. It was, however, found by a preliminary experiment that the presence of glycine did not affect the time course of the reaction of diphenethynyl mercury and urease protein as revealed by the spectrophotometric experiment.

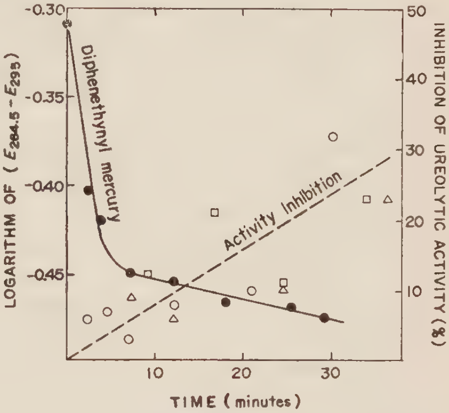

Fig. 7. Course of reaction of diphenethynyl mercury and crystalline urease as followed by change in concentration (expressed in absorbancy) of diphenethynyl mercury and change in degree of inhibition of ureolytic activity of urease.

Reaction system: 0.113 g. per liter urease (82,400 units per g. of protein), 1.03×10^{-5} *M* diphenethynyl mercury, 0.05% glycine in *M*/50 phosphate buffer (pH 7.0) containing 30% (v/v) aqueous ethanol. Control system (used also as reference solution) contained no diphenethynyl mercury. Concentration of diphenethynyl mercury is expressed in terms of ($E_{264.5}$-E_{295}) as explained in text (●). At this concentration of urease, nearly 10^{-5} *M* sulfhydryl groups are expected to be present based on calculation using sulfhydryl number of 44 per molecule of urease (*2*, *5*).

Estimation of ureolytic activity: an aliquot was taken out and subjected to estimation of enzyme activity (3.5 minutes at 25°C). Results of triplicate experiments are indicated (○, △, □).

As plotted in Fig. 7, logarithm of ($E_{264.5}$-E_{295}) representing the concentration of diphenethynyl mercury, dropped abruptly at the period of initial 8 minutes followed by slow decrease. It should be mentioned that even after 30 minutes of reaction period, almost 70 per cent of diphenethynyl mercury was found to remain unreacted. Therefore the occurrence of apparently slow reaction process in this case may not the result of the exhaustion of diphenethynyl mercury in this reaction system. These observations suggest the presence of at least two processes in the reaction of diphenethynyl mercury with urease protein.

The course of change in the degree of inhibition of the ureolytic activity is also plotted in the same figure. Thus inhibitory activity increased almost linearly at time proceeded, although the activity was exhibited only partially.*

From such observations, it is highly probable, as postulated earlier by Hellerman (*2*), that in the urease molecule there are sulfhydryl groups highly reactive with mercurials and independent on the ureolytic activity of this enzyme, and that sluggish sulfhydryl groups involving in the ureolytic activity are also present in this enzyme molecule. The rapid reaction process demonstrated in Fig. 7 may be the reflection of the reaction of diphenethynyl mercury with reactive sulfhydryl groups in urease molecule. Slow but steady reaction of this mercurial with sluggish sulfhydryl groups also seems to occur, resulting in the loss of ureolytic activity of this enzyme. The fact that the inhibitory activity of this mercurial on urease was exhibited only partially so far observed suggests that the reactivity of the sluggish sulfhydryl groups with diphenethynyl mercury may only be limited.

SUMMARY

1. The stoichiometric studies on the reaction of diphenethynyl mercury and glutathione were carried out spectrophotometrically. It was found that 1 to 2 moles of the mercurial and glutathione, respectively, gave 1 mole of diglutathionyl mercury and 2 moles of phenylacetylene. These final prodocts were always found to be produced, independent of the initial molar ratios of these two reactants.

2. Kinetical studies performed on this reaction system revealed that a first-order reaction was rate-limiting process on the molecule of diphenethynyl mercury and that more complicated reaction process occurring on glutathione molecule. Possible reaction schemes on this reaction system are presented.

3. The spectrophotometric method was also applied to the study on the reactivity of diphenethynyl mercury with urease protein. It was concluded that diphenethynyl mercury reacted easily with "reactive" sulfhydryl groups of urease protein, whereas it reacted slowly with "sluggish" ones. This view was further supported by the fact that diphenethynyl mercury exhibited only a partial inhibition on the ureolytic activity of urease; the cite of enzymatic activity of this enzyme has been known to be involved in the reactivity of the "sluggish" sulfhydryl groups in the protein molecule.

The author wishes to express his deep gratitude to Prof. T. Yanagita of Chiba University and Prof. A. Takamiya of University of Tokyo for their kind guidance and encouragement throughout this work. Thanks are due to Mr. M. Matsui, Director, and Dr. Y. Miura of this Laboratory for their kind encouragement, and to Dr. S. Osaki of Tokyo Institute of Technology for his technical guidance in ultracentrifugation.

The author is indebted to Messrs. T. Onoe, O. Amakasu, and H. Higuchi, and to Misses C. Furukawa and H. Ohtsuka of this Laboratory, for elemental analyses and infrared spectral measurements.

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The Journal of Biochemistry, Vol. 50, No. 2, 1961

L-Lysine Oxidase, a New Oxygenase

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(Received for publication, February 6, 1961)

Suda and his colleagues (*1*, *2*) have described the enzyme L-lysine oxidase, which was isolated from a strain of *Pseudomonas* and which specifically oxidized L-lysine according to the following equation:

L-Lysine + $O_2 \longrightarrow \delta$-Aminovaleric Acid (AVA)*** + CO_2 + NH_3

This enzyme was purified about 200 fold. It was shown that the oxygen consumption and the formation of the three products were equimolar throughout the reaction period; that neither pyridine nor flavine nucleotides participated in the reaction and that the addition of catalase and electron accepting dyes had no effect on the rate of the reaction.

The properties of this enzyme and its mode of action seemed to be very similar to those of lactic oxidative decarboxylase which was isolated by Sutton from *Mycobacterium phlei* (*3*) and which catalyzed the following reaction:

$$\text{Lactic acid} + O_2 \longrightarrow \text{Acetic acid} + CO_2 + H_2O$$

Hayaishi and Sutton (*4*) found that this latter enzyme was an oxygenase which utilyzed molecular oxygen.

This fact raises the possibility that L-lysine oxidase might also be an oxygenase. The present report shows that, when L-lysine was oxidized by the oxidase in the presence of O^{18}-labeled gas, at least one atom of oxygen in the product was derived from molecular oxygen, and that L-lysine is indeed an oxygenase.

EXPERIMENTAL

Enzyme Preparation—The strain of *Pseudomonas* and the method of preparation of the acetone dried powder has been described elsewhere (*1*).

Three g. of the acetone dried powder were homogenized with 120 ml. of water and extracted for 1 hour at 0°C. After centrifugation, 2.4 ml. of 1 *M* $MnCl_2$ were added to the supernatant. This mixture was centrifuged and the supernatant was dialyzed overnight against distilled water. The precipitate was discarded after centrifugation and the supernatant was used as the enzyme solution. The pH was kept at 7.4 throughout the preparation and the temperature at around 4°C.

O^{18} Tracer Experiments—The incubation mixture (100 ml.) consisted of; 60 ml. of enzyme solution, 4 millimoles of Tris buffer (pH 8.0), and 250 mg. of L-lysine-HCl, contained in a flask of approximately 500 ml. volume specially designed for O^{18}-experiments (*5*). The contents of the flask were frozen by immersion in a dry ice-ethanol bath, the flask evacuated and then allowed to thaw. After complete thawing, the contents were refrozen and the flask again evacuated. O^{18}-Labeled oxygen and nitrogen gas were admitted separately into the vessel in an approximate ratio of 1 : 4. A small quantity of this gas mixture was removed for mass spectrometric analysis as initial gas sample. The temperature was then brought to 37°C. The reaction was started by injecting 5 ml. of L-lysine solution through a rubber stopper and allowed to proceed at 37°C with gentle shaking. At intervals, aliquots of the solution were removed and the reaction was followed by measuring the ammonia liberated in the solution. After a 4 hour incubation period (when the reaction was complete), the contents of the flask were frozen and a final gas

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*** The abbreviations used are: δ-aminovaleric acid, AVA; Tris, trishydroxyaminomethane.

sample was removed for analysis.

H_2O^{18} experiments were carried out by the same method except that all solutions were made up with enriched H_2O^{18} and the gas phase contained unlabeled oxygen and nitrogen gas. The enzyme solution in H_2O^{18} was prepared by dissolvidg the lyophilized residue in the same volume of H_2O^{18} as the original volume of natural water solution.

Isolation of AVA—The isolation of crystalline AVA-HCl was as reported previously (*2*), except that all isolation steps were carried out at around 4°C and the volume was reduced by lyophilization. The crystals so obtained were dissolved in a small amount of methylcellosolve and recrystallized by the addition of ether.

Determination of the O^{18} Abundance Ratio—In order to determine the O^{18} abundance ratio in the reaction products, they were pyrolyzed in a sealed, evacuated pyrex tube by the method of Rittenberg and Ponticorvo (*6*) using $HgCl_2$ as a catalyst, and the CO_2 thus produced was introduced into a Consolidated Electrodynamic Cooperation Mass Spectrometer model 21-410. The O^{18} abundance ratio was calculated from the ratio of recorded peak heights corresponding to the ion currents of $C^{12}O^{16}O^{16}$ and $C^{12}O^{16}O^{18}$. The initial and final gas samples were measured by admitting them directly into the inlet system of the spectrometer in order to determine their components, constitution and isotopic abundance ratio. The O^{18} abundance in water was measured by the gaseous oxygen prepared by electrolysis in a specially designed U-tube.

RESULTS AND DISCUSSION

The oxidation of L-lysine was carried out under conditions in which oxygen in the gas phase was labeled with O^{18} and oxygen in the aqueous phase was natural isotopic abundance. The O^{18} abundance ratio in the AVA formed were measured as described above. It can be seen in Table I that the O^{18} abundance ratio in the AVA produced under O^{18} labeled oxygen gas is significantly higher than that in AVA produced under unlabeled oxygen gas, and the latter agreed completely, as was expected, with the natural abundance in CO_2. Experiment II is a duplication of Experiment I. One of the reaction products, CO_2, was detected in the final gas samples, and its O^{18} abundance ratio was the same as that from ordinary AVA, indicating therefore, that the carboxyl carbon of L-lysine had been released.

These results show that the reaction L-lysine—AVA involves the fixation of molecular oxygen, although there was probably oxygen exchange between O^{18} in the enzymatically formed AVA and O^{16} in the medium so that the figures obtained were values which had been considerably diluted during the reaction and isolation procedures. Thus one or two atoms of molecular oxygen should have been enzymatically incorporated whereas the actual incorporation of O^{18} was lower, less than one atom (see Table I).

TABLE I

Enzymatic Incorporation of Molecular Oxygen

Exp.	Atom % excess			%
	In O_2		In AVA[1] formed	$\frac{\text{Found}}{\text{Expected}^{2)}} \times 100$
	Initial	Final		
I	0.940	0.940	0.243	51.8
II	0.920	0.920	0.185	39.8

The experimental conditions are described in the text.

1) The abundance ratio in AVA produced under normal oxygen was 0.204%.

2) Expected if one oxygen atom in AVA was derived from the molecular oxygen.

In order to decide whether an exchange of oxygen atoms had actually occurred and if so, how much exchange had occurred during the reaction, O^{18}-labeled AVA was reisolated under conditions in which the temperature, pH, time, and the concentration of the components were just the same as those of Table I. The O^{18}-labeled AVA was prepared by heating a solution of AVA-HCl in H_2O^{18} in a sealed pyrex tube at 100°C for 24 hours. This labeled sample was well dried *in vacuo* for several days before use. Table II illustrates the results obtained in duplicate experiments. From these data, it can be concluded that about half of the oxygen atoms exchanged. However, considerable disagreement existed between the observed values and this prevented an accurate correction. No exchange during recrystalization from organic solvents was detectable, as seen in Table III. It is, therefore, likely that the exchange reaction occurred during deproteinization with perchloric acid, undoubtedly because of the acidity of the solution. As shown in Table IV hydrogen ions accerated the exchange of O^{18} between AVA and H_2O. It seems likely that the carboxyl oxygen of AVA is involved (*7*).

TABLE II

Dilution of O^{18} Abundance Ratio in AVA Isolation

Exp. No.	Atom % excess in O^{18}-AVA		% $\frac{\text{Found}}{\text{Original}}\times 100$
	Initial	Isolated	
I	1.138	0.768	67.5
II	1.138	0.499	43.8

The experimental conditions are descriced in the text.

TABLE III

Exchange of O^{18}-AVA during Crystallization

Recrystallized from lized	Times recrystallized	Atom % excess	
		Initial AVA	Recrystallized AVA
Ethanol-ether	1	1.40	1.43
	4	//	1.45
Methylcellosolve-ether	1	//	1.40
	4	//	1.39

O^{18}-AVA was prepared as in Table II. Ethanol was dried with Mg, ether with Na and distilled. Methylcellosolve was purified by fractional distillation. 0.130 g. of O^{18}-AVA HCl was dissolved in 1 ml. of ethanol (or methylcellosolve), filtered, and 10 ml. of ether was added. After 30 minutes, crystals were collected by centrifugation and dried under vacuum more than 24 hours. All the manipulations were carried out at 4°C.

It seems clear that the abundance ratio of enzymatically formed AVA in the reaction mixture must have been greater than that in the isolated compound, and that this enzyme acts as an oxygenase, incorporating at least one atom of molecular oxygen.

TABLE IV

Oxygen Exchange between AVA and H_2O

Exp. No.	Medium	Atom % excess	% $\frac{\text{Found}}{\text{Original}}\times 100$
O^{18}-AVA	orginal	1.29	—
I	1N HCl	0.018	15.1
II	pH 2.5	0.83	64.3
III	pH 4.5	1.16	97.5
IV	pH 10.6	0.758	63.9

O^{18}-AVA HCl was dissolved in *N* HCl (Experiment I), in distilled water (Experiment II), in *N* ammonia (Experiment III), or in distilled water and its pH adjusted by adding conc. ammonia (Experiment IV). The final concentration of AVA was *M*/5. After 24 hours at 22°C, the reaction mixture was lyophilized and the O^{18} abundance ratio was measured as described in the text.

Table V shows the results of an experiment where the water phase was labeled with O^{18}. After termination of the reaction, the reaction mixture was immediately lyophilized

TABLE V

Oxidation of L-Lysine in H_2O^{18}

Exp. No.	Atom % excess		% $\frac{\text{Found}}{\text{Expected}}\times 100$
	In H_2O^{18}	In AVA	
I	1.66	0.149	18.0a)
II	1.66	0.022	2.6b)

In Experiment I, L-lysine was incubated with the enzyme in H_2O^{18}. In Experiment II, AVA was incubated with the enzyme in place of L-lysine.

a) Expected if one oxygen atom in AVA was derived from water.

b) Expected if one oxygen atom in AVA exchanged with water.

to remove H_2O^{18} and then dissolved in natural water. Thus the enzymatically produced AVA was exposed to H_2O^{18} during the incubation, but to natural water during the isolation procedures. In Experiment I, 18 per cent incorporation was observed from enriched H_2O^{18}. To test whether this incorporation was due to an enzymatic reaction or to chemical exchange during the reaction, the nonenzymatic exchange was measured in a control experiment (Experiment II) which was carried out in parallel with Experiment I, using AVA in the presence of the enzyme in enriched H_2O^{18}. In the control experiment, the conditions were identical to those of Experiment I except that AVA was incubated in place of L-lysine. The concentration of AVA in Experiment II was equivalent to that of L-lysine in Experiment I, in which AVA was formed enzymatically. If none of the oxygen atoms in the AVA had come from water, the incorporation in Experiments I and II should have been equal. In the control experiment, however, there was only 2.6 per cent incorporation.

From the above data, it can not be decided definitely whether one atom of oxygen in AVA originates from H_2O or not, because the dilution of O^{18} abundance ratio in the isolation procedure changed each time and an accurate correction for the exchange reaction is not possible. However, the results indicate clearly that L-lysine oxidase acts as an oxygenase in the oxidation of L-lysine to AVA, fixing molecular oxygen into the product.

SUMMARY

1. When L-lysine was oxidized by L-lysine oxidase to δ-aminovaleric acid in a medium in which the molecular oxygen was labeled with O^{18}, at least one atom of molecular oxygen was incorporated into the product isolated from the reaction mixture.

2. A chemical exchange between the carboxyl group and the oxygen in the medium resulted in a dilution of O^{18} abundance ratio of the enzymatically produced δ-aminovaleric acid.

3. When the enzymatic oxidation of L-lysine was performed in a medium containing H_2O^{18}, an apparent incorporation of O^{18} into δ-aminovaleric acid was observed. It is possible that this was an artifact due to the exchange reaction occurring during the isolation procedure.

This investigation was supported in part by a research grant from the National Institutes of Health, United States Public Health Service (C-4222) and a Scientific Research Grant from the Ministry of Education of Japan to one of us (O. H.).

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The Journal of Biochemistry, Vol. 50, No. 2, 1961

The Chemistry of Lipid of Posthemolytic Residue or Stroma of Erythrocytes

X. Acetone-Soluble Lipids of Human Erythrocytes

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(Received for publication, February 8, 1961)

Earlier communications from this laboratory have dealt with the isolation and characterization of acetone-insoluble lipids, *viz.*, glycolipids (*1–8*) and phospholipids (*1*, *5*, *6*, *9*) of mammalian erythrocytes. However, very little is as yet known of the chemical nature of acetone-soluble lipid except the presence of cholesterol in very high concentration, even though they are of particular interest in relation to erythrocytes metabolism and function.

This report describes the extraction and fractionation of these lipids by silicic acid chromatography combined with infrared measurement and chemical analyses. As the results, the presence of triglyceride and esterified cholesterol as well as a relatively large amount of free fatty acids were confirmed. The chemical compositions of these individual lipids are given, including the identification and estimation of their fatty acid component.

In addition, so-called "oxy-cholesterol" was obtained, though the sample was not so large in amount that its characterization was only unsatisfactory.

EXPERIMENTAL

Preparation of Material—Pooled acetone-soluble parts from ether-methanol extract of human erythroytes in our previous experiments (*5*, *8*, *9*) were used as the starting material. Human erythrocytes were hemolyzed with six volumes of 0.3% acetic acid solution, and the stroma precipitated was spun down by means of continuous centrifugation, washed and freeze-dried. The dried stroma was extracted with ether-methanol (1 : 1) at room temperature for three days. After four such extractions, the solution was concentrated *in vacuo*, to which about five volumes of acetone were added. Thus obtained acetone solution was concentrated *in vacuo* and a large amount of cholesterol was permitted to crystallize out, which was then removed by filtration. After repeating this procedure, large parts of cholesterol were removed. The mother liquor, from which no more crystals separated out, was concentrated to a syrup and dissolved in petroleum ether, transferred into a rubber sac and subjected to dialysis against a large volume of the same solvent by the procedure recommended by Van Beers *et al.* (*10*). By this treatment, only acetone-soluble lipids passed through the membrane. This procedure seems more profitable, particularly when the amount of sample was large, than the silica column technique of Borgström (*11*) or Barron and Hanahan (*12*). After changing the outer fluid once a day three times, the pooled diffusate were concentrated *in vacuo* and applied to a silicic acid column by the procedure of Barron and Hanahan (*12*).

Analytical Procedures—Phosphorus was determined by Allen's (*13*) and iodine value by Yasuda's method (*14*). For optical rotation measurement, Hitachi spectrophotometer EP-U-2A was used. Infrared determination was performed with a Hitachi EP-I-2, double-beam automatic recording spectrometer with samples pressed in KBr or as liquid film.

Fatty Acid Determination—Reversed-phase column chromatographic procedure of Silk and Hahn (*15*) was used for hydrolysate and its hydrogenated product. In order to determine the nature and amount of fatty acids, calculation was done for each value (*7*). The initial fraction eluted by 45% acetone was apparently lower than capric acid but its nature was undetermined (*7*).

RESULTS

In a typical experiment, 4.8 g. of light yellow semi-solid material obtained from 270 g.

of dried stroma was loaded on a column of 200 g. of silicic acid (Mallinckrodt, 100 mesh, for chromatography) which was pre-washed with ether, benzene and hexane (*12*). Elution was carried out with 1,800 ml. of hexane, 500 ml. of 15% benzene-hexane, 1,800 ml. of 10% ether-hexane, 1,800 ml. of 20% ether-hexane and 1,500 ml. of pure ether, successively. Fractions of each 100 ml. of solution leaving the column were collected, evaporated to dryness under reduced pressure and weight of the residue was determined. As illustrated in Fig. 1, an elution curve was obtained which may indicate rough separation of components into four peaks.

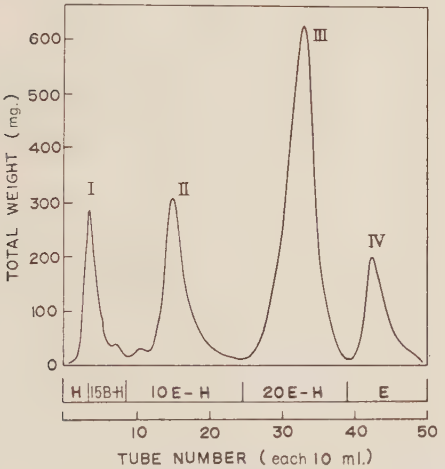

Fig. 1. Chromatography of acetone-soluble lipids of human erythrocytes. The solvents were hexane (H), benzene (B), and diethyl-ether (E). See text.

The first fraction (*I*)—This was semi-solid and apparently an esterified cholesterol (442 mg.) as determined by its infrared absorption spectra. Iodine value : 60.64. Eighty eight mg. of this material was refluxed with ethanolic KOH on a boiling water-bath but complete hydrolysis was not so easy to attain and required prolonged period. The non-saponifiable matter was identified as cholesterol by chemical and infrared analysis as well as by optical rotation measurement. Fatty acid components were: lower than capric 4%, palmitic 46%, stearic 16%, oleic 7% and linoleic (or palmitoleic) 27%.

The second fraction (*II*)—This fraction (915 mg.) was likely a mixture of triglyceride and free fatty acids. As has been indicated, these two components were hardly separated by ordinary silicic acid chromatography (*11*, *12*), but the separation was accomplished by the use of 'Silica Gel' of Kanto Chemicals & Co. Six hundred and ninety mg. of the mixture was dissolved in 10% ether-hexane and applied to a column of "Silica Gel" (30 g.).

Triglyceride—By eluting with 10% ether-hexane, 153 mg. of triglyceride free of non-esterified fatty acid was obtained. Iodine value, 54.26; C 72.73, H 11.92, Glycerol 11.85. By hydrolysis of 64 mg. of the material with aqueous baryt, 49 mg. of fatty acids mixture was obtained, which proved to consist of the following acids: lower than capric 24%, myristic 7%, palmitic 39%, stearic 13%, oleic 10% and linoleic (or palmitoleic) 7%. From the aqueous solution, after removal of barium with sulfuric acid, concentration and benzoylation, glycerol tribenzoate was isolated and identified by means of infrared measurement.

Free Fatty Acids—After triglyceride was eluted, 356 mg. of free fatty acids devoid of triglyceride was obtained by elution with ether. (Iodine value 68.1). The components were separated by reversed phase chromatography before and after hydrogenation. The results were: lower than capric 12%, palmitic 47%, stearic 23%, oleic 12% and linoleic (or palmitoleic) 6%.

Fraction III—Although large proportion of free cholesterol had been removed by crystallization, its small part remained and appeared in this fraction (1.95 g.).

Fraction IV (912 mg.)—According to Barron and Hanahan (*12*) di- and monoglyceride appeared in these areas. Though these fractions gave positive acrolein test, considerable amount of non-saponifiable materials was obtained after hydrolysis with ethanolic KOH. For the separation of these substances, chromatography on Brockmann's alumina according to the procedure of Ruzicka's school (*16*, *17*) was used.

Eight hundred mg. of non-saponifiable material was dissolved in hexane and loaded on 20 g. of Brockmann's alumina (without activation). Elutions were carried out with 100 ml. of hexane, 100 ml. of benzene, 85 ml. of ether and 80 ml. of 10% methanol-ether successively and finally with 40ml. of methanol. Separation into roughly five fractions can be seen from the effluent curve (Fig. 2).

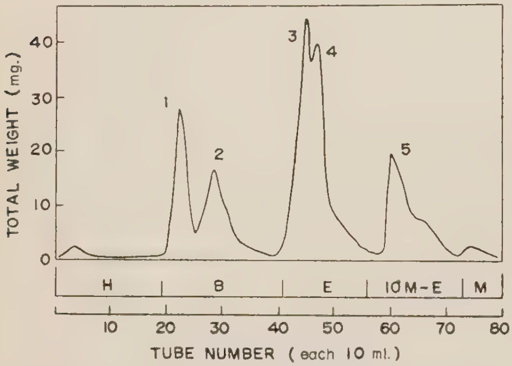

Fig. 2. Chromatography on alumina of the non-saponifiable matter of human erythrocytes. The solvents were hexane (H), benzene (B), ether (E) and methanol (M).

'Fraction 1' (60.7 mg.) was crystalline and after recrystallization from methanol-water gave small platelet melting at 112–4°C. In view of infrared spectra (Fig. 3A) (*18*), analytical data and chromatographic behavior (*16*, *17*), this material was most probably cholesta-3,5-dien-7-one.

Calcd. for $C_{27}H_{42}O$, C 84.75, H 11.07
Found 84.99, 10.90

$[\alpha]_{589}=-258^\circ$ (in chloroform), $\lambda_{max}=277 m\mu$ ($\varepsilon=15,100$ in ethanol). The results by Ruzicka *et al.* (*17*) were: m. p. 114°C, $[\alpha]_D=-305^\circ$ (in chloroform), $\lambda_{max}=277 m\mu$, while by Cook (*19*, p. 98) $\lambda_{max}=280 m\mu$, ($\varepsilon=24,400$).

It was suggested by infrared analysis that 'Fraction 2' (110 mg.) was cholesterol which had not yet been completely discarded.

Since the separation of 'Fraction 3 and 4' was incomplete, they were subjected to re-chromatography on alumina. The anterior 'Fraction 3' (111.5 mg.) was, nevertheless, non-crystalline and further purification by means of rechromatography and treatment with Girard-reagent to give crystalline compound was without success. Infrared (Fig. 3B) (*18*) and ultraviolet spectra ($\lambda_{max}=240 m\mu$) indicated this material to be cholest-5-en-3-β-ol-7-one (7-ketocholesterol).

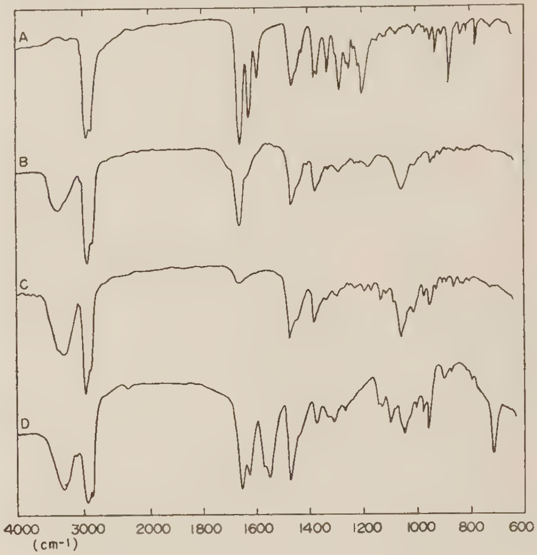

Fig. 3. Infrared spectra of non-saponifiable matters from human erythrocytes. A. Fr. 1, (cholesta-3,5-dien-7-one), B. Fr. 3, (7-ketocholesterol), C. Fr. 4, (7-hydroxy cholesterol), D. Fr. 5, ceramide.

Calcd. for $C_{27}H_{44}O_2$, C 80.93, H 11.08
Found // 81.16, // 11.65

For comparison, an authentic specimen was prepared from the autoxidation mixture of colloidal cholesterol solution by the procedure of Bergström and Wintersteiner (*20*). The infrared spectrum, chromatographic behavior and ultraviolet maximum (240 mμ) of this synthetic crystalline material, m. p. 170–2°C, were in good agreement with those from erythrocytes.

The posterior 'Fraction 4' (124.4 mg.) became crystalline and after recrystallization from methanol-water gave platelet melting at 149–153°C. Further recrystallization did not change the melting point. $[\alpha]_{589}=-42^\circ$ (in chloroform).

Calcd. for $C_{27}H_{46}O_2$, C 80.54, H 11.51
Found // 79.27, // 11.38

From the infrared spectrum (Fig. 3C),

analytical value and positive Lifschütz reaction (*17–20*), it would probably be cholest-5-ene-3-β-7-diol (7-hydroxycholesterol). The results of previous authors for this diol were m.p. 188°C, $[\alpha]_D=-95°$ for 7α-, and m.p. 178°C, $[\alpha]_D=+7°$ for 7β-epimer (*19*), while different values, *viz.*, m.p. 157°C, $[\alpha]_D=-90°$ (for 7α) is taken up in another monograph (*18*) and the material from hog spleen (*17*) gave m.p. 168–170°C, $[\alpha]_D=-12.3°$, which was at that time described as 7α, but is 7β according to the present rule of nomenclature (*19*, p. 59).

Though the physical constants of erythrocytes material deviate from those reported previously, it would possibly be a mixture of α- and β-epimer of 7-hydroxy-cholesterol.

'Fraction 5' (95.4 mg.) proved to be a ceramide, *viz.*, acyl (mostly lignoceryl)-sphingosine by infrared measurement (Fig. 3D). This material was also detected in the non-saponifiable material of hog spleen (*17*).

DISCUSSION

There have been reported a number of works on erythrocytes lipids concerning their quantitative estimation and distribution on micro-scale. Among them, the reports of Thannhauser and Setz (*21*) on ox red cells, Erickson *et al.* (*22*), Hack (*23*) on human red cells and Burt and Rossiter (*24*) on rabbit erythrocytes are notable. But their main interest seemed to be in complex lipids other than acetone-soluble fraction, which represents only a very small proportion of total lipid except cholesterol. Since we were in a favorable condition to have a large amount of source material as a by-product of isolating complex lipids, attempt to characterize this fraction was made. Nevertheless, the amount of lipids other than cholesterol was so small that the accurate study, especially of non-saponifiable material, could not be accomplished. The yield of individual lipid from two experiments are summarized in Table I. Since the data shown in the Table are based on the real weight of isolated material, they might be somewhat smaller than those given by quantitative determination, but they indicate that the components other than cholesterol are very minute in amount. It was reported that cholesterol ester was not contained in rabbit erythrocyte (*24*) and according to other workers (*22*), its amount varied from zero to 1.1 per cent between animal species. In this study, the presence of cholesterol ester and the nature of its fatty acid components were established. They were shown to be relatively saturated, while those from serum cholesterol ester comprise, as is well known, poly-unsaturated fatty acids.

TABLE I

Yields of Each Acetone-Soluble Lipid from Human Erythrocytes Stroma

(As percentage of dried material)

Substance	Run I	Run II
Total lipid	ca. 20	
Cholesterol	4.0	4.2
Cholesterol ester	0.27	0.16
Free fatty acids	0.50	0.13
Triglyceride	0.054	0.057
'Oxy-cholesterol'	0.20	0.34

As for the triglyceride and free fatty acids of erythrocyte, there has also been no report and it is a matter of some dispute whether the relatively large amount of free fatty acids observed in one Experiment (Run I) might be formed during the isolation process, but it is certain that it was not yielded by hydrolysis on silicic acid chromatography, as demonstrated by control experiment using pure triglyceride, though the possibility that it might be an artefact could not be excluded.

The presence of 'oxy-cholesterols' has been said to be the result of autoxidation of cholesterol during isolation and purification. However, the characteristic pattern of their occurrence as summarized in Table II might indicate that these materials are not artefacts but occur *in situ*. Nevertheless, 3,5-cholestadiene-7-one is thought to be formed from alkaline treatment from 7-keto-cholesterol.

TABLE II

Distribution of 'Oxy-cholesterol' in Various Organs

Substances	Serum (*25*)	Liver (*26, 27*)	Testis (*28*)	Aorta (*16*)	Spleen (*17*)	Erythrocytes
1. Cholest-4-ene-3β-6-diol					+	
2. Cholest-5-ene-3β-7β-diol	+	+			+	+
3. Cholest-5-ene-3β-7α-diol	+	+				
4. Cholest-6-ene-3β-5-diol					+ ?	
5. Cholestane 3β, 5α, 6β-triol		+	+	+		
6. Cholest-4-en-3-one			+		+ ?	
7. Cholesta-4,6-dien-3-one					+	
8. Cholesta-3,5-dien-7-one			+	+	+	+
9. Cholestan-3β-ol-6-one					+	
10. Cholest-5-en-3β-ol-7-one			+			+

SUMMARY

1. Acetone-soluble lipid was isolated from a large amount of human erythrocytes and was fractionated by silicic acid column chromatography.

2. It was separated into cholesterol, cholesterol ester, triglyceride, free fatty acids and non-saponifiable matter.

3. Chemical nature and amount of component fatty acids were determined by reversed-phase chromatography.

4. Non-saponifiable matter other than cholesterol were divided by alumina column, from which cholesta-3,5-dien-7-one, 7-keto-cholesterol and 7-hydroxy- cholesterol were detected.

The authors wish to express their gratitude to Dr. M. Kitano of Nihon Blood Bank & Co. for the supply of human blood cells.

The expenses for the work was aided by the Scientific Fund furnished by the Ministry of Education, for which the authors wish to thank.

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The Journal of Biochemistry, Vol. 50, No. 2, 1961

On the Initial Stage on the G-F Transformation of Actin

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(Received for publication, February 27, 1961)

We have shown that the G-F transformation of actin caused by the addition of salts is to be regarded as a condensation phenomenon (*1*). With the increase in the protein concentration at a constant ionic strength, globular actin (G-actin) molecules are converted into the fibrous form (F-actin) above a certain critical concentration and then the amount of F-actin is linearly increased.

Now a problem arises concerning the steps through which the actin is polymerized. Two postulations on the actin polymerization have been proposed; one by Szent-Györgyi (*2*) and the other by Tsao (*3, 4*). According to the former view based on the electron micrographs of F-actin, the unit of F-actin filament, named "ovoid", is the G-actin aggregate of about 100Å in width and 300Å in length. However, the existence of such ovoids in solutions has not yet been ascertained. The latter view is based on the experimental results obtained by polarized fluorescence and osmotic pressure measurements. From these measurements, Tsao concluded that the actin existed in the two forms, monomer and dimer, and the kinetic unit in F-actin was the actin dimer, although the long fibrous structure of F-actin had been observed in various experiments, e. g., electron microsopy, flow birefringence, and light scattering etc. The presence of such a kinetic unit in F-actin may be due to the rotational freedom of the actin dimer around long axis of F-actin. However, the monomer-dimer picture does not seem to be so decisive.

It is probably sure that G-actin molecules in salt-free solutions are separated from each other by the electric repulsion and as the charge effect is diminished by the addition of salt or by the adsorption of ions, the polymerization occurs. In the intermediate steps of the polymerization, certain aggregates are observed as was described in the previous paper (*5*). Below the critical actin concentration, we saw that the apparent molecular weight of G-actin became larger with the addition of a small amount of magnesium ion. This means that the G-actin tends to associate with each other, forming small aggregates. The appearance of such small aggregates may be regarded as a pre-condensation phenomenon from the standpoint of interpreting the G-F transformation to be a condensation phenomenon.

On reflecting these situations, it is necessary to investigate the initial stage of actin polymerization more thoroughly. The best method of such an investigation is the light scattering because of its high sensitivity in detecting small aggregates. The results obtained by this method are reported here.

EXPERIMENTAL

Materials—The crude G-action solution extracted from the acetone dried rabbit skeletal muscle was prepared according to Feuer *et al.* (*6*), and it was purified mainly by the Mommaerts procedure (*7*). Some modification was made in the step of the second precipitation of F-actin, where we dispersed the F-actin pellet obtained from the first polymerized F-actin solutions into 0.06 *M* KCl and spun down to remove lipids and inactive actin.

Since the final G-actin solutions, after dialysis against water containing a small amount of adenosine triphosphate (ATP) ($<10^{-4}M$), comprised some traces of F-actin which were not deploymerized during dialysis, they were centrifuged at 100,000×g for about 3 hours. The supernatant solutions collected carefully were our original G-actin solutions. Generally, they contained more than 75% polymerizable active actin.

Protein concentrations (C_A) were conventionally determined by the refractive index increment, measurements of which were made with a differential refractometer.

The samples to be measured were made up by adding the original solutions to aqueous solvents containing veronal-HCl buffer (pH 8.0) and neutral salts of various concentrations. After standing at the room temperature for about one hour in order to accelerate the rate of attaining equilibrium state, they were stored at 0°C, since it took a week or longer to attain equilibrium state only on storage at a low temperature, and actin gradually lost its polymerization activity.

Measurements—Light scattering measurements were carried out by a Shimazu light scattering photometer with a light of wave length 436 mμ from a mercury arc. Small cylindrical cells were used. Each solution was clarified by centrifugation at $20{,}000 \times g$ to remove large particles such as dusts or flocculates prior to the measurement.

The refractive index increment dn/dc determined by the differential refractometer was 0.177, which is in agreement with that obtained by Mommaerts (*8*).

The calibration of turbidity was made by the use of standard liquids, benzene and toluene, the Rayleigh ratios of which were taken 48.5×10^{-6} and 58×10^{-6} respectively. The corrections necessary for the calibration with standard liquids were made according to Carr and Zimm (*9*, *10*). By these procedures, we obtained the molecular weight of serum albumin (bovine) as 82,500, which is higher than that usually reported (*10*). This value seems to be satisfactory, however, since small aggregates may be present in the solution of serum albumin (*11*).

The other correction factors in the determination of molecular weight, depolarization and dissymmetry, were measured. The depolarization factor of G-actin (ρ) was 0.03; hence, the Cabbanes factor, $6+6\rho/6-7\rho$, became 1.06. The dissymmetry, the ratio of the scattering intensity at angle 45° to that at 135°, was between 1.1 and 1.3 in the solution of G-state. This dissymmetry factor was taken into consideration in the determination of the molecular weight; however, the reduced intensity at 90°, R_{90}, was used in most of the discussions.

Sedimentation experiments were made with a Spinco Model E Analytical Ultracentrifuge.

THEORETICAL

Our system is constituted of three components; one solute, protein (subscript 2) and two species of solvents, salt (subscript 3) and water. According to the theory of light scattering from multi-component systems (*12*), the turbidity τ from such a system can be written in the form:

$$\tau-\tau_0=(8\pi^3/3\lambda^4)(\partial n^2/\partial C_2)^2_\mu\, a_{33}/(a_{22}a_{33}-a_{23}), \qquad 1$$

where n is the refractive index of the system, λ is the wave length of incident beam, C_2 is the concentration of the macro-ions in number, μ is the chemical potential, τ_0 is the turbidity from solvents, and a_{22}, a_{23} and a_{33} are the activity derivatives with respect to the concentrations. Since the macro-ions are small compared with the wave length of the incident beam, we rewrite the equation by replacing $\tau-\tau_0$ with R_{90}:

$$R_{90}=(2\pi/\lambda^4)(\partial n/\partial C_2)^2 n^2 a_{33}/(a_{22}a_{33}-a_{23}). \qquad 2$$

This equation is quite similar to that derived by Edsall *et al.* (*10*). The only difference is in the expression of the refractive index increments.

When the activity derivatives with respect to the concentrations are expressed by the Scatchards formulation, and when $ZC_2 \ll 2C_3$, the final result is written as follows:

$$KC_2/R_{90}=1/M+1/M^2(Z^2/2C_3+\alpha)C_2,$$
$$\alpha=\beta_{22}-\beta_{23}{}^2C_3/(2+\beta_{33}C_3), \qquad 3$$

where C_3 is the concentration of salt in number per unit volume, C_2 is the protein concentration in g. per unit volume, β_{22} is the activity coefficient derivative with respect to the protein concentration, β_{23} and β_{33} are those with respect to the salt concentration, Z is the number of net charge on the protein, M is the molecular weight of the protein, and $K=(2\pi/\lambda^4)(dn/dC_2)^2n^2$. The first term in the parentheses of the virial coefficient is the so-called Donnan term and the second term includes the interaction between protein molecules, β_{22}, that between salt and protein, β_{23}, and that between salt molecules, β_{33}. This equation is useful for analysing the interactions between various components.

RESULTS

The molecular weight of G-actin in salt free solutions can be obtained by the extrapolation of KC_A/R_{90} to zero concentration as shown in Fig. 1. On account of the large

Donnan effect, the curve of KC_A/R_{90} *versus* C_A is not expressed by a straight line but shows a sharp initial increase followed by a slower increase. Such a behavior at very low salt concentrations is naturally expected from the equation 3. The molecular weight obtained by the extrapolated intercept of KC_A/R_{90} is 117,000, which is nearly twice as much as the monomer molecular weight usually reported, 57,000 (*8*). That is to say, our G-actin is dimer. This molecular weight is not changed by a further addition of small amount of ATP, about $10^{-4}M$, while the slope of the curve is decreased with a tendency to become linear, as shown in Fig. 1.

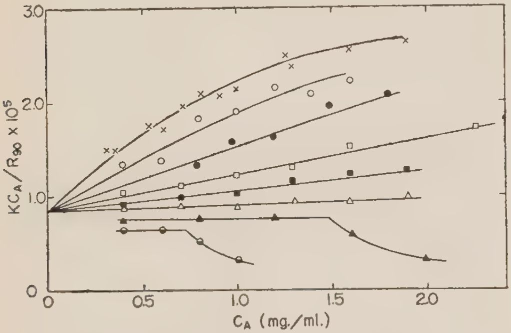

FIG. 1. The concentration dependence of KC_A/R_{90}, as a function of salt concentration. The concentration of buffer denotes C_B and that of KCl C_K. Veronal-HCl buffer, pH 8.0. × : $<10^{-4}M$ ATP, ○ : ATP$=10^{-4}M$, ● : $C_B=1$ mM, □ : $C_B=3$ mM, ■ : $C_B=5$ mM, △ : $C_B=7$ mM, $C_K=5$ mM, ▲ : $C_B=7$ mM, $C_K=10$ mM, ◒ : $C_B=7$ mM, $C_K=15$ mM.

When the buffer and KCl are added to the G-actin solutions, the slope of the KC_A/R_{90} *versus* C_A curve has a smaller value with increasing ionic strength, whereas the extrapolated intercept remains constant, the inverse of which still gives the dimer molecular weight, 117,000. Finally at a certain critical salt concentration, the curve becomes parallel to the horizontal line. In the range of the salt concentration smaller than this critical salt concentration, C_{sc}, the G-F transformation does not occur even at high protein concentrations.

When the salt concentration exceeds the critical value C_{sc}, the flat part of the curve at low actin concentrations begins to take a value lower than that corresponding to the dimer; i.e., the apparent molecular weight of G-actin (M_{ap}) observed below the critical actin concentration C_{cr} becomes greater than that of the dimer. When the actin concentration is increased at such a salt concentration, a sudden decrease of KC_A/R_{90} is observed at C_{cr}, where the G-F transformation begins to take place. With the increase in the salt concentration, M_{ap} becomes larger and the value of C_{cr} becomes lower. These results are shown in Fig. 1.

When $MgCl_2$ is employed as the polymerization reagent, a similar result to that with KCl is obtained. Fig. 2 shows the relation between KC_A/R_{90} and C_A at various magnesium concentration [Mg]. No G-F transformation is observed below a certain critical magnesium concentration $[Mg]_{cr}$, (in the figure, about 0.3 mM). An increase in the magnesium concentration does not alter the extrapolated molecular weight which is equal to that of the dimer, but makes the virial coefficient small. Above $[Mg]_{cr}$, M_{ap} increases and C_{cr} decreases with the increase in magnesium concentration and the polymerization can take place.

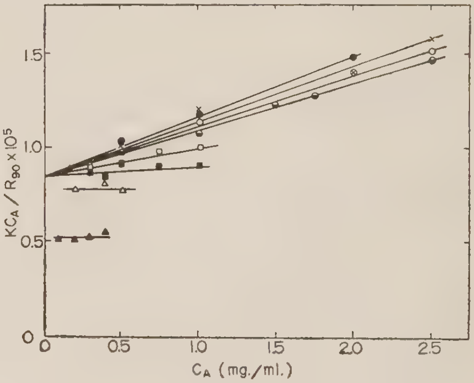

FIG. 2. The relation of KC_A/R_{90} *versus* C_A at various magnesium concentrations [Mg]. Veronal-HCl buffer, 3 mM, pH 8.0. ● : [Mg]$=0.1$ mM, × : [Mg]$=0.15$ mM, ○ : [Mg]$=0.2$ mM, ◒ : [Mg]$=0.25$ mM, □ : [Mg]$=0.3$ mM, ■ : [Mg]$=0.35$ mM, △ : [Mg]$=0.4$mM, ▲ : [Mg]$=0.5$ mM.

The above results are summarized by the relations of M_{ap} and the virial coefficient A to the salt concentration as shown in Fig. 3.

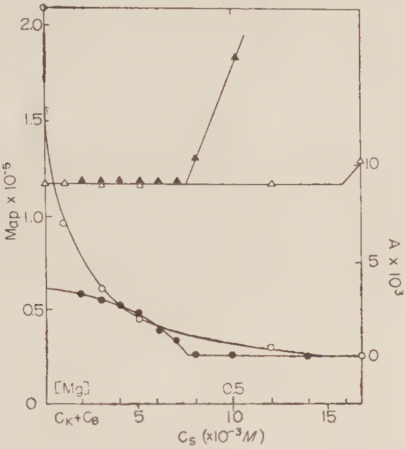

FIG. 3. The dependence of M_{ap} and the virial coefficient, A, on the salt concentration. The same abbreviation as in Figs. 1 and 2 is used. △ : M_{ap} (C_B and C_K), ▲ : M_{ap} ([Mg] with 3 mM C_B). ○ : A (C_B and C_K), ● : A ([Mg] with 3 mM C_B).

As the salt concentration is increased, A decreases, whereas M_{ap} is kept constant at a value of 117,000 so far as C_s is below C_{sc}. At C_{sc} where A is reduced to zero, M_{ap} begins to increase. Therefore, the reduction of the charge effects by the addition of salts seems to be an essential requirement for the occurance of the G-F transformation.

Above C_{sc}, namely, a condition, under which the G-F transformation can take place, M_{ap} becomes larger with the increase in the salt concentration. In order to make clear this initial stage of the G-F transformation, the turbidity from the solutions is plotted against C_A (Figs. 4 and 5). In these figures, the typical G-F transformation can be observed by the break of the curves at C_{cr}, the slope of which in the dilute region gives M_{ap}. It is noticed that the turbidity at C_{cr}, $R_{90,\,C_{cr}}$, is found to lie on a straight line as indicated by the broken line in Figs. 4 and 5. Therefore, when we increase the actin concentration under a certain salt concentration higher than C_{sc}, the turbidity of the solution is increased linearly with C_A, and the G-F transformation begins at the point where the line of R_{90} *versus* C_A meets with the line of $R_{90,\,C_{cr}}$ shown by the broken line in the figures.

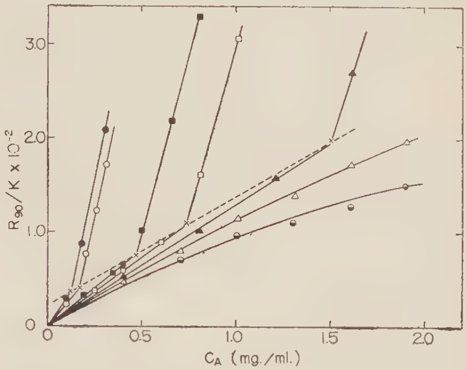

FIG. 4. Plots of R_{90} *versus* C_A to show the relation between $R_{90,\,C_{cr}}$ and C_{cr}. The same abbreviation as in Fig. 1 is used. Veronal-HCl buffer, pH 8.0. ◒ : C_B=5 mM, △ : C_B=7 mM, C_K=5 mM, ▲ : C_B=7 mM, C_K=10 mM, □ : C_B= 7 mM, C_K=15 mM, ■ : C_B=7 mM, C_K=20 mM, ○ : C_B=7 mM, C_K=30 mM, ● : C_B=7 mM, C_K= 40 mM.

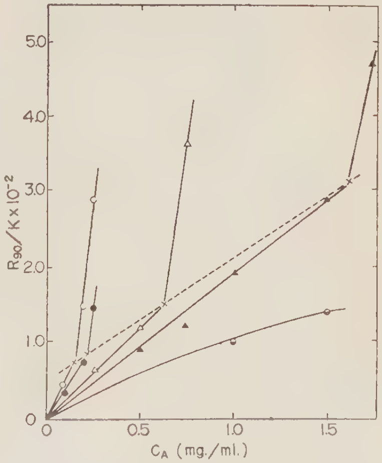

FIG. 5. Plots of R_{90} *versus* C_A to show the relation between $R_{90,\,C_{cr}}$ and C_{cr}. The same abbreviation as in Fig. 2 is used. Veronal-HCl buffer, pH 8.0, 6 mM. ◒ : [Mg]=0 mM, ▲ : [Mg]=0.4 mM, △ : [Mg]=0.6 mM, ● : [Mg]= 0.8 mM, ○ : [Mg]=1.0 mM.

In the solutions before the occurrence of the G-F transformation, the presence of some aggregates is evident as shown by the increase of M_{ap}, but it is not easily concluded whether all the G-actin molecules or some fraction of them take part in the polymerization. However, if all the G-actin molecules are aggregated to polymerized molecules having a molecular weight of M_{ap}, it is difficult to understand the condition of the F-actin formation at C_{cr}, since these aggregates become larger with the increase in the salt concentration. For the analysis of this result, therefore, we tentatively assume that a part of G-actin molecules are polymerized into small aggregates and their number increases linearly with the increase in the actin concentration up to the critical concentration C_{cr} of the gel-like F-actin formation. Namely, the increase in M_{ap} is assumed to be due to the partial polymerization of G-actin molecules into small aggregates and not to their uniform growth. In addition, we assume that regardless of salt concentration, a constant concentration of polymerized molecules is necessary for the formation of the gel-like F-actin, which begins at C_{cr}. On these assumptions, the turbidity at the critical actin concentration under different salt concentrations must depend linearly on C_A and the slope of this line must give the molecular weight of G-actin coexisting with polymerized molecules in the solutions of the initial stage, since the turbidity from the polymerized molecules at C_{cr} is constant. Therefore, these assumption are fit for the explanation of the results. A value of about 120,000 for the molecular weight of G-actin coexisting with the polymerized molecules obtained from the slope makes it sure that these assumptions are quite reasonable.

A support to the above assumptions is given by the results of sedimentation experiments. Since the concentration range of the initial stage is comparatively narrow and is limited to a dilute region, we carried out the measurements at a concentration low enough in the desired range and high enough for observing the main peak, neglecting the concentration dependence of the sedimentation constant. Sedimentation constants ($C_A=0.2$ mg./ml., 5 m*M* Tris buffer, pH 8.0) with and without 0.6 m*M* [Mg] are found to have the same value of about 3.5 S. This suggests that the main part of the solution is not changed by the addition of magnesium ions. Thus, it is possibly concluded that the increment of the turbidity below the critical actin concentration of the G-F transformation, i.e., a greater apparent molecular weight than that of G-actin, is due to a small amount of polymerized G-actin molecules and the increase in the number of such polymerized molecules.

Fig. 6 shows that M_{ap} above the critical magnesium concentration increases linearly with [Mg]. This result, with those described previously, makes possible the explanation of the hyperbolic relation between [Mg] and C_{cr} (*13*) as in the following. When we denote the molecular weight of G-actin as M_G and that of polymerized actins found below C_{cr} as M_f, and their concentrations as C_G and C_f respectively, M_{ap} is expressed as follows;

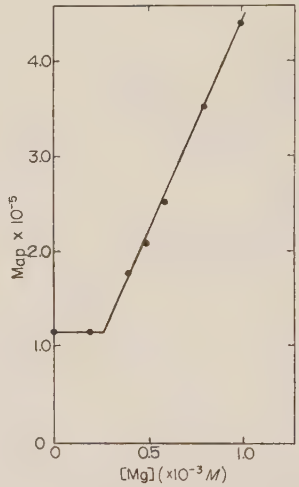

FIG. 6. M_{ap} as a function of [Mg].

$$M_{ap}=(C_GM_G+C_fM_f)/C_G+C_f. \qquad 4$$

As described previously, M_{ap} increases linearly with [Mg] above $[Mg]_{cr}$;

$$M_{ap}=M_G+a([Mg]-[Mg]_{cr}), \qquad 5$$

where *a* is a proportional constant. Therefore, at the critical actin concentration, the following equation is obtained from equations 4 and 5;

$$C_{cr}([Mg]-[Mg]_{cr})=C_f(M_f-M_G)/a. \qquad 6$$

Since we can put both C_f and M_f to be constant following the previous conclusion, the right hand side of equation 6 is independent of the salt concentration. This shows that the relation between C_{cr} and [Mg] is hyperbolic.

DISCUSSION

It was examined whether the decrease of virial coefficient with the increase in a salt concentration would be attributable to the change of the refractive index increment, since dn/dc may give serious effects on turbidity. However, no change in dn/dc was observed by the addition of salts in our experimental range; therefore we can reasonably conclude that the experimental results are accounted for by the depression of the charge effects.

It must be due to the adsorption of magnesium ions by actin molecules that a smaller amount of magnesium ions than expected by the estimation from the ionic strength are very effective to the depression of the virial coefficient. Because the Donnan term in the virial coefficient, $Z^2/2C_3$, includes the square of the charge on the molecule, the decrease in the charge by the binding of salt ions makes the virial coefficient effectively small. It is, of course, expected that the monovalent small ions would also be adsorbed by the protein, and therefore the exact estimation of the extent of adsorption is very difficult. On the basis of the Donnan term and the adsorption phenomenon, the effects of pH and the species of buffers on the G-F transformation can be qualitatively explained. The net charge on the actin molecule depends not only on pH but also on the adsorption capacity of buffer ions. With the decrease in pH and with the increase in the adsorption capacity, both the virial coefficient and the critical concentration become smaller.

At the critical salt concentration, the virial coefficient is reduced to zero. This means that the interaction term, α, in the equation 3 is negative, since the Donnan term still remains positive at the critical salt concentration. Namely, an attractive force between actin molecules is balanced with the charge repulsion. In actin solutions, the critical salt concentration, where the curve of KC_A/R_{90} to C_A becomes flat, is comparatively low and therefore a considerably high repulsion is present between actin molecules. This indicates that a high attractive force is necessary for the polymerization; that is to say, a simple precipitation of a common protein corresponds to the polymerization of actin. As a result, it is suggested that both repulsive and attractive forces are involved in the polymerization mechanism of actin as was pointed out by Mommaerts (*8*). In order to estimate the attractive force, we assume that at the critical concentrations of KCl and $MgCl_2$, the attractive force is of the same order. Since the number of Mg ions adsorbed by each actin is about 5 (*13*), we can obtain the interaction term and the protein charge from the experimental data as $\alpha=-14,000$ and $Z=19$, by putting the critical concentration of salts, $C_A=14\,\mathrm{m}M$ and $[\mathrm{Mg}]=0.35\,\mathrm{m}M$ respectively.

It must be examined whether the polydispersity in the original solution may have an effect on our molecular weight of G-actin, 117,000, higher than that of the monomer usually accepted. In fact, the original solutions may occasionally include some polymers, however, these polymers must be depolymerized with dilution, and then the extrapolation to infinite dilution must give the particle weight of the unit of actin. Otherwise, diverse molecular weight would be obtained in every preparation, since different amounts of polymers would be contained in each solution. Furthermore, in such a case, a sufficient centrifugation would make the value smaller. However, these tendencies have never been observed and the molecular weight ofther dimer is always obtained. Therefore, we have to conclude that the kinetic unit of G-actin defined physically has a molecular weight, 117,000. Since one ATP is bound to one actin of the moleular weight, 57,000 (*14*), the physical unit of G-actin has two ATP molecules. When each ATP is dephosphorylated at the conversion of G-actin to F-form, it is expected that each G-actin has two polymerization sites. This suggests that F-actin has the possibility of forming a network structure.

The molecular weight of G-actin above described is discrepant with those obtained by Mommaerts (*8*) and by Kay (*15*). This seems to be due to the difference of experimental condition such as the experimental range of protein concentration and to that of sample. In our experiments, the protein concentration was taken from 0.3 mg./ml. to 2 or 3 mg./ml., lower than those in their experiments, for the purpose of making precise extrapolation to infinite dilution. Otherwise, an initial sharp increase in the curve of KC_A/R_{90} at very low salt concentration might have been overlooked. In the experiment of Kay, the discrepancy would also be due to the difference of sample, probably inclusion of inactive actin in his preparation, which is suggested by the data of depolarization of zero and its low helical content; according to the preliminary results of optical rotatory dispersion, active actin has about 35 per cent of helical content.

As was described previously, the increase of M_{ap} before polymerization is due to the increase in the number of the polymerized molecules. However, in extremely dilute solutions the aggregates must dissociate into unit G-actins. If this is true, the curve of KC_A/R_{90} should rise upwards with dilution to the value which would give the dimer molecular weight. Because of the limit of the experimental technique, however, we could not find the tendency of this dissociation.

The role of dephosphorylation of ATP observed in the G-F transformation has been a common interest for every worker in the field of musculer contraction. It is not readily concluded, however, whether or not the dephosphorylation would directly be involved in the polymerization mechanism, that is, ATP on G-actin molecules would directly relate to the origin of the attractive force. (*16*)

SUMMARY

The initial stage of actin polymerization caused by KCl and $MgCl_2$ was studied mainly by means of the light scattering. Below a certain critical salt concentration, the molecular weight of G-actin is a constant value of dimer, 117,000, regardless of the salt concentration, while the virial coefficient becomes smaller with the increase in the salt concentration. These results indicate that the unit of active actin is a dimer, having two polymerization sites, and that the depression of charge effects is an essential condition of the G-F transformation. Above this critical salt concentration, the G-F transformation begins at a critical actin concentration. Below this critical concentration of actin, the apparent molecular weight is greater than that of the dimer. This suggests that some parts of actin have already polymerized to a certain degree before the initiation of the G-F transformation; when the concentration of these polymerized actins reaches a critical value, then G-F transformation starts.

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The Journal of Biochemistry, Vol. 50, No. 2, 1961

Iodine Metabolism in Pig Thyroid Slices and Effect of Thyrotropic Hormone

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(Received for publication, March 13, 1961)

It is well known that thyroid tissue slices accumulate iodide and convert it to protein-bound iodine as thyroid glands do *in vivo*. As to the effects of thyrotropic hormone (TSH), studies were made by several investigators using radioactive iodine as a tracer. Until now, various kinds of stimulation have been reported as TSH effects in slices: they are, promotion of collection and release of iodide (*1*), enhanced formation of proteinbound iodine from I^{131}-iodide (*2*, *3*), and increase in the total I^{131} uptake (*4*, *5*). However, some workers could not obtain the similar results or even found inhibiton by the addition of TSH (*6–8*).

As the fate of iodine is not simple in thyroid glands, the results of tracer experiments should be interpreted with caution. Thus, it is conceivable that, increased radioactivity in a given component (for instance, protein-bound iodine) caused by the addition of TSH does not necessarily mean that TSH enhances the true rate of iodine transfer to the component in question. On the contrary, if it is assumed that the action of TSH is not unifocal, an apparent decrease in labeling might be observed even though stimulation of the steps under consideration is really occurring. These considerations show that more fundamental studies on the intrathyroidal iodine metabolism are required to elucidate the mechanism of TSH action.

In the present study, re-examination of various phases of intrathyroidal iodine metabolism in the presence or absence of TSH was carried out making use of I^{131} as well as I^{127} determinations in a hope that these experiments might clarify the ever-obtained contradictory results and provide useful information concerning the action of TSH.

EXPERIMENTALS

Preparation of Thyroid Slices and Incubation—Pig thyroid glands were obtained at a slaughter-house and kept on ice until sliced. Within 2 hours after the animals were killed, thyroid slices, 0.5–0.7 mm. thick, were prepared with a razor blade, and rinsed with an ice-cold buffer. The buffer used was a Krebs-Ringer phosphate solution (pH 7.2) in which concentration of $CaCl_2$ was reduced to a half. Following this rinse, 100–200 mg. of slices were introduced to a 20 ml.-beaker. Incubation medium contained 0.5 μc of carrier-free NaI^{131}, KI and the Krebs-Ringer solution mentioned above, in a total volume of 3.0 ml. The concentration of KI was kept at 0.05 μg. I per ml., unless otherwise stated. In experiments designed to test the effect of TSH, 100 μg. of TSH preparation was added to the incubation medium.

Incubation was perfomed in a shaking incubator at 37°C in the air. Incubation time, which varied with experiments from 15 minutes to 3 hours, will be described later with results of each experiment. At the end of incubation, the slices were taken out, rinsed with the buffer, and transfered to a beaker containing 3 ml. of boiling ethanol to stop the reaction. In experiment in wich electrophoretic or chromatographic examination was performed, the incubated slices were treated in different manners (see each section).

Determination of Protein-Bound I^{131}—Following the incubation, the slices suspended in 3 ml. of ethanol were homogenized in a Potter-Elvehjem glass homogenizer and the homogenate was filtrated to obtain the ethanol-insoluble protein fraction. The insoluble protein was washed with ethanol and its radioactivity was determined in a well-type scintillation counter.

Determination of Iodide in Slices—The clear filtrates containing iodide were combined, and the volume was adjusted to 15 ml. with ethanol. The radioactivity was determined using 2 ml. of the solution. The rest of the solution was diluted with two volumes of distilled water and applied to a Dowex-3 (OH-type) column (0.6 cm. in diameter and 10 cm. in height). Following washing of the column with 30 ml. of 50% aqueous ethanol and 30 ml. of distilled water, iodide adsorbed to the resin was eluted with *N* NaOH. Iodide content was determined* by a modified Grossmann's method (*9*) using an appropriate portion of the eluate. When fresh slices were used to determine the iodide content, carrier-free NaI^{131} with known radioactivity was added to the homogenate and the recovery of iodide in the chromatographic operation was calculated.

Determination of I^{131}-Iodide in Incubation Midium—After incubation, 3 ml. of 20% trichloroacetic acid solution was added to the incubation medium. After removal of the precipitated proteins, the supernatant was applied to a Dowex-3 column, and treated as described before.

Chromatographic Examination of Enzymatic Digest of the Labeled Slices—After incubation, the rinsed slices were transferred to a beaker containing 1 ml. of the buffer and boiled for 5 minutes to stop the reaction. The slices thus treated were homogenized in a Potter-Elvehjem glass homogenizer, and the homogenate was digested with "Pronase" at 37°C for 24 hours. 0.01–0.02 ml. of the digest was applied on a paper strip (Toyo Filter Paper No. 50) and chromatographed at room temperature for 18–24 hours, using a solvent system composed of *n*-butanol-acetic acid-water (78 : 5 : 17, in V : V : V).

Chromatographic Identification of Organic Iodine in the Incubation Medium—The mediums in five beakers were combined after incubation and extracted with an equal volume of acid butanol (0.1 *N* HCl-*n*-butanol (1 : 9)). The extract separated from the protein fraction remaining in the aqueous phase was concentrated at approximately 40°C under stream of the warm air. The concentrated extract was chromatographed using the solvent system described before.

Paper Electrophoresis—Following incubation, the slices were treated with the ice-cold buffer containing 0.07 *M* thiourea and homogenized in a Potter-Elvehjem glass homogenizer. 0.01–0.02 ml. of the homogenate was applied to a paper. Electrophoresis was carried out in a veronal buffer (pH. 8.6, $\mu=0.075$) at constant current density of 0.45 mA/cm. at room temperature for 5–10 hours. After electrophoresis, the paper was dried at 110°C and stained with bromophenol blue to localize the components.

Detection and Measurement of Radioactive Components Separated on the Paper—Radioactive components on the paper were located by radioautogram after exposure of the paper strip to Fuji X-ray film for an appropriate period. For quantitative estimation of the labeled components on the paper, the appropriate area of the paper was cut out and counted in a well-type scintillation counter.

Materials—TSH was prepared from frozen whale pituitaries by a method (*10*) essentially similar to the method of Ciereszko (*11*). Biological activity of this preparation was approximately 1 U. S. P. unit/mg. "Pronase" (*Streptomyces griseus* protease) (*12*) was kindly furnished by Dr. M. Nomoto.

RESULTS

Conversion of Iodide to Protein-Bound Iodine in Thyroid Slices—When pig thyroid slices were incubated with NaI^{131}, fairly large amount of I^{131} was incorporated into the slices. The radioactivity of iodide in the slices increased rapidly during the initial period of the incubation, reached a maximum within 20—30 minutes, and maintained, thereafter, nearly a constant value. On the other hand, the labeled organic iodine increased more slowly than iodide, but the increase was found to last at least 2—3 hours (Fig. 1). In another experiment, slices were incubated with NaI^{131} for 10 minutes, and then labeled slices were re-incubated in the medium without containing radioactive iodide. In the second incubation, labeled protein-bound iodine was found to be formed rapidly from labeled iodide in

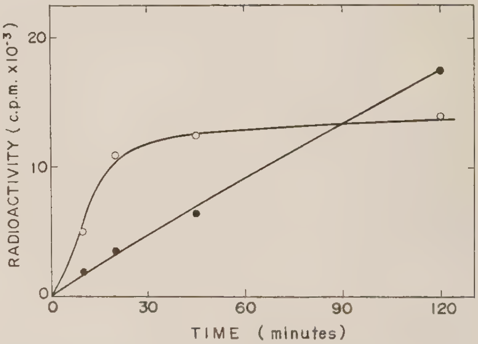

FIG. 1. Time course of labeling of iodide and protein in thyroid slices.

—○— Iodide. —●— Protein-bound iodine. Incubation was performed at 37°C under shaking in the air.

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* To be published.

the slices (Fig. 2).

From these results, it is suggested that, in agreement with *in vivo* finding, iodide in medium is incorporated into slices and then, in turn, converted to protein-bound iodine.

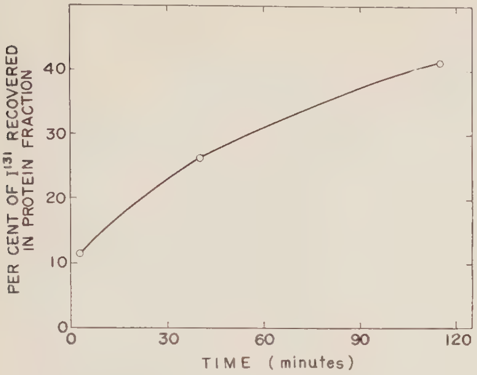

Fig. 2. Formation of protein-bound iodine from iodide accumulated in slices.

Thyroid slices were pre-incubated with NaI^{131} for 10 minutes and the labeled slices were incubated for various period in isotope-free medium.

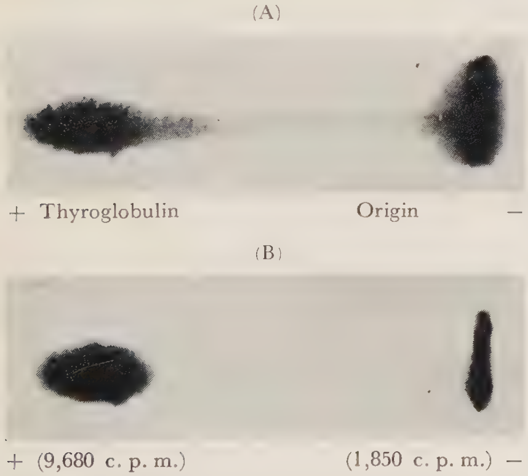

Fig. 3. Electrophoretic diagram of labeled proteins in slices after incubation.

(A) Electrophoretic diagram stained with BPB.

(B) Radioautogram.

Characterization of Protein-Bound Iodine in Slices—In order to identify the protein into which incorporation of I^{131} occurred, the slices which had been incubated with labeled iodide for 15 minutes were examined by paper electrophoresis. As shown in Fig. 3 (A), the electrophoretic diagram stained with bromophenol blue showed that the main component was thyroglobulin. The second component remained at the origin was thought to be proteins in particulates of the cells. Other protein components were also detected in the area between the thyroglobulin spot and the origin, but their amounts were negligibly small. As shown in the radioautogram of the same strip (Fig. 3 (B)), most of the radioactivity was recovered in the thyroglobulin area, and the activity remained at the origin was less than 20 per cent of the total activity. Accordingly, it leaves no doubt that protein-bound iodine formed during incubation of slices exists chiefly in thyroglobulin molecules. The fact agrees with *in vivo* finding.

The newly formed iodine compounds in thyroglobulin molecules were identified as iodinated amino acids from the following chromatographic experiments. The paper chromatogram of the homogenate of the incubated slices showed that radioactivity was completely recovered in the origin (protein) and iodide. When the labeled slices were digested enzymatically and chromatographed, monoiodo- and diiodotyrosines, besides I^{131}-iodide, were detected on the chromatogram

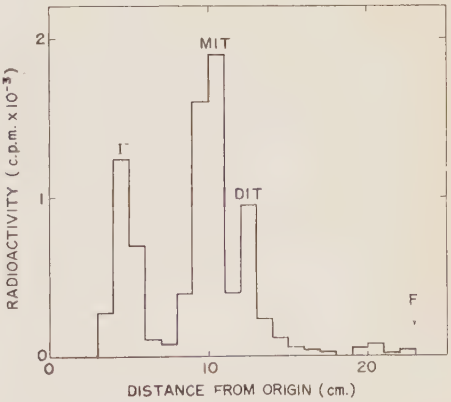

Fig. 4. Incorporation of I^{131} into monoiodo- and diiodotyrosines. Figure shows the distribution of radioactivity in paper chromatogram of the enzymatic digest of slices incubated with NaI^{131} for 2 hours. I^-: iodide, MIT and DIT: mono- and di-iodotyrosine, F: solvent front.

(Fig. 4) in agreement with other investigators' observations. In some cases, an additional radioactive spot was found close to the solvent front, although its contribution to the total activity was less than a few per cent. This component seems to be "unknown component (UK_1)" previously reported by other workers (*3*, *18*).

Release of Organic Iodine from Slices—It was found that, after a prolonged incubation, several per cent of the total radioactivity in the incubation medium existed as organic compounds with lower molecular weights. In order to identify the labeled compounds, the butanol-HCl extract of the incubation medium obtained after 3 hours' incubation was examined by paper chromatography. As shown in Fig. 5, three radioactive comonents found on the paper chromatogram were identified as monoiodo- and diiodotyrosines and iodide from their R_f values. Weakness in the radioactivity of iodide spot, seen in the figure, would be due to the loss of iodide during the extraction of the medium with butanol-HCl. Besides these components, about one third of the total radioactivity was found to distribute to the area between diiodotyrosine spot and the solvent front; the position of maximum radioactivity varied between diiodotyrosine area and solvent front from experiment to experiment. The radioactive components were thought to be iodothyronines and "unknown comonent (UK_1)."

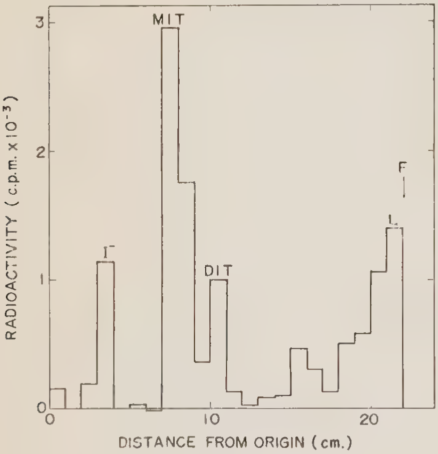

FIG. 5. Demonstration of organic iodine compounds in incubation medium.

Figure shows the distribution of radioactivity in paper chromatogram of the *n*-butanol-HCl extract of the incubation medium after 3 hour's incubation. I^-: iodide, MIT and DIT: mono- and di-iodotyrosine, F: solvent front.

Release of Iodide from Thyroid Slices—It was also observed that iodide, besides organic iodine, is released from slices into medium. The fact was confirmed from the following two observations. At first the specific radioactivity of iodide in the incubation medium fell with the duration of incubation (see Fig. 10), and even the net increase in iodide content in the medium was observed occasion-

TABLE I

Release of I^{131}-Iodide from Thyroid Slices Labeled in vitro

	I^{131}-iodide (c.p.m.)	
	in slices	in medium
Before re-incubation	40,000	—
After re-incubation	18,000	14,800

First incubation: 10 min. Re-incubation: 40 min.

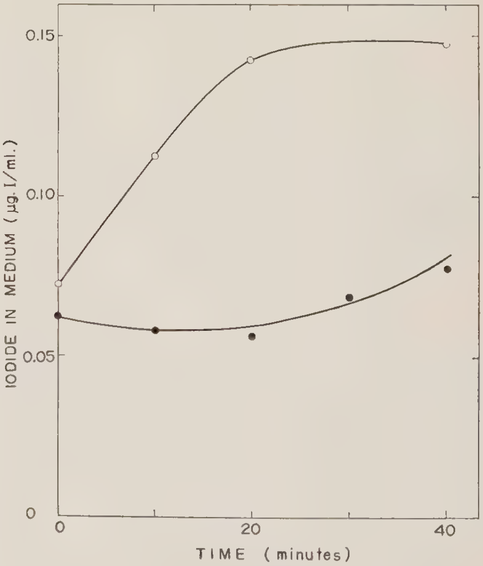

FIG. 6. Change in iodide concentration in incubation medium.

—○— Experiment 1. —●— Experiment 2.

ally (Fig. 6). Secondly, when the labeled slices obtained after incubation with NaI^{131} were re-incubated in an isotope-free medium, fairly large portion of the radioactivity in the medium was found to be iodide (Table I).

Effects of TSH on the I^{131} Incorporation into Slices—Thyroid slices were incubated in the presence or absence of TSH, and total I^{131} uptake and I^{131} incorporation into ethanol-insoluble (protein-bound iodine) fraction were determined. Each typical time course is illustrated in Fig. 7.

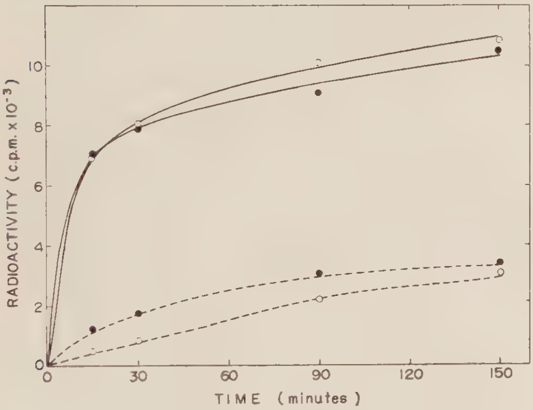

Fig. 7. Time course of I^{131} incorporation into thyroid slice in the presence (—●—) and absence (—○—) of TSH.

(—) Total uptake. (- - -) Incorporation into protein.

Total I^{131} uptake by the slices was often not influenced by the addition of TSH, unless incubation was prolonged over 1 to 2 hours. On the other hand, as shown in this figure, the radioactivity of the protein fraction was higher in the presence of TSH than in the absence during the initial period of the incubation. The difference in the radioactivity of the protein fraction caused by TSH diminished gradually with time. In some instances, the amount of I^{131} incorporated into the protein fraction became less in the presence of TSH than in the absence of TSH when the incubation was prolonged.

Several these experiments had two results in common: 1. In the earlier period of incubation, the I^{131}-iodide incorporation into protein fraction was definitely stimulated by TSH while the total I^{131} uptake by the slices was scarecely altered by TSH. 2. The rate of incorporation of I^{131}-iodide into protein fraction decreased more rapidly in the presence of TSH than in the absence.

Iodide Pool in Thyroid Gland and Its Stability—A tracer experiment which makes use of I^{131}-iodide is, no doubt, very useful for investigating the transfer of iodine in thyroid slices. However, it should be pointed out that mere comparison of radioactivity would be meaningless for the purpose of comparison of reaction rates under different conditions, unless the changes in the pool sizes of metabolic intermediates were negligibly small. If the changes are appreciable, it is indispensable to estimate pool size or specific radioactivity of the intermediate. From this point of view, attempts were made to estimate pool size* of iodide and its behavior during incubation. As shown in Table II, iodide pool size (stable iodide content in thyroid) varied

Table II

Variation of Iodide Content of Pig Thyroid Gland

Gland No.	Iodide content (mμg. I/100 mg. of wet tissue)
1	73
2	91
3	95
4	117
5	125
6	130
7	140
8	261
9	267
10	447
11	762
12	818
13	1,490

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* In this estimation, it was assumed for the sake of convenience that thyroid slices contained only one kind of iodide pool.

considerably from gland to gland. However the pool size in a given gland was found to remain almost constant, or rather to increase, after a prolonged incubation at 37°C (see Fig. 9). The same result was obtained with incubation at 0°C (see Fig. 9); in this case, I^{131}-iodide in the medium was scarcely incorporated into the slices.

In contrast with this finding which obtained when the concentration of iodide in medium (0.05 μg./ml.) was lower than that in slices, the iodide content in slices considerably increased by incubation in iodide-rich medium. The extent of the incrdase was found to depend upon the iodide concentration in the medium. Fig. 8 shows the result of the experiment in which iodide concentration was varied from 0.05 to 5 μg. I per ml. of medium and incubation was performed for 20 minutes.

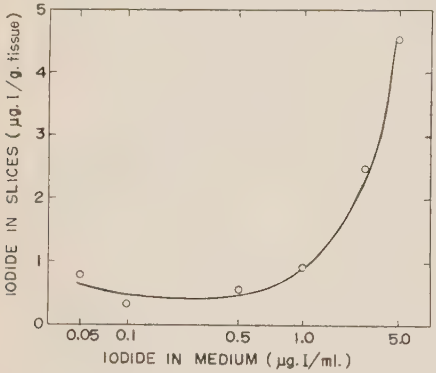

Fig. 8. Increase in iodide content in slices after incubation in iodide-rich medium.

Effect of TSH on Iodide Pool—Thyroid slices were incubated with I^{131}-iodide in the presence or absence of TSH and the change in stable iodide content or specific radioactivity was studied. The incubation of slices with TSH did not cause an appreciable fall in iodide content in the slices, but caused rather a slight increase (Fig. 9). The change in specific radioactivity of iodide in slices and in medium are illustrated in Fig. 10.

These results suggest that stimulatory effect of TSH on I^{131} incorporation observed in the standard incubation medium (see Fig. 7) is really the enhancement of the rate of conversion of iodide to protein-bound iodine. It is also suggested that the extent of this stimulatory effect would be underestimated if merely I^{131} incorporation rates are used.

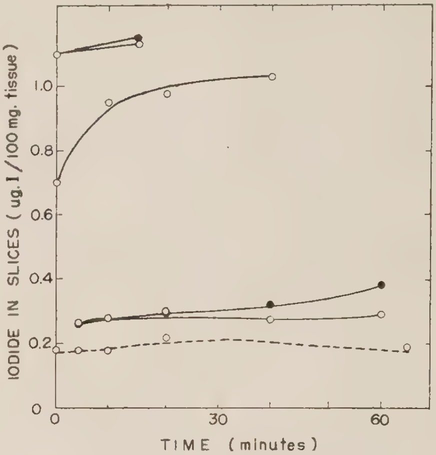

Fig. 9. Change in iodide content in slices during incubation at 37°C (—) and at 0°C (---). —○— No addition. —●— Plus TSH.

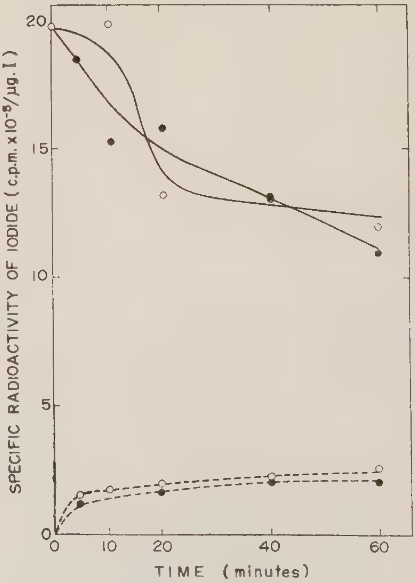

Fig. 10. Change in specific radioactivity of iodide in slices (---) and in medium (—), in the presence (●) and absence (○) of TSH.

DISCUSSION

The results described above indicate that almost all phases of intrathyroidal iodine metabolism *in vivo* can be found in surviving tissue slices. Although essentially the same conclusion has been obtained by other investigators (see (*13*)), the results of the present study based on analyses of both I^{131} and I^{127} provides more extented knowledges concerning the metabolism of iodine in slices and the role of TSH.

The presence of iodide-concentrating mechanism in slices are clear from the experiments described in this paper. However, it is to be noted that, in spite of the presence of the active transport of iodide, the iodide (I^{127}) content in thyroid slices does not change appreciably during the incubation if iodide concentration in medium is not much higher than that in plasma (0.01-0.05 μg./ml. in rat (*14*)). The constancy of the pool size of iodide in slices is, probably, due to balancing of the rates of iodide accumulation, iodide release to medium (Table I, Figs. 1 and 6), intrathyroidal conversion of iodide to protein-bound iodine and of deiodination of iodinated organic compounds. The presence of the iodide (I^{127})-release from slices causes the fall of specific radioactivity of iodide in medium during the course of incubation (Fig. 10). Accordingly, it is clear that rate of I^{131} incorporation into slices is not directly proportional to the true rate of iodide transfer from the incubation medium. Recently, the release of iodide from thyroid gland has been demonstrated also *in vivo* (*15—17*). The finding that the iodide concentration in medium even increased with incubation time (Fig. 6) suggests that deiodination of iodinated organic compounds occurs in slices.

The conversion of iodide to protein-bound iodine, the second process of the iodine metabolism, was found to occur slowly but persist through the incubation. Newly formed protein-bound iodine was proved to be localized in the protein fraction as monoiodotyrosine or diiodotyrosine in agreement with the results obtained by other workers (*3*, *18*). In addition, this iodoprotein was identified as thyroglobulin as it is so *in vivo* though less than 20 per cent of the total protein-bound I^{131} was found in particulate fraction (*19*, *20*). The formation of labeled thyroxine within slices has been scarcely observed; it is probably due to the dilution of labeled precursors with stable ones. However, it is to be noted that the rate of iodide conversion to protein-bound iodine (R_p) is calculated, as will be shown later, to be an order of 0.001-0.01 μg./min./100 mg. of wet tissue, and the values qualitatively agree with the reported secretion rate of thyroxine in animals (order of 100 μg./day in man (*13*)).

The presence of labeled organic iodine, such as moniodotyrosine and diiodotyrosine in the incubation medium is rather curious, as monoiodotyrosine or diiodotyrosine is not released from intact glands, probably due to deiodination of iodotyrosines by intrathyroidal deiodinase. Accordingly, the release of iodotyrosines might show the lack of integrity of iodine-transfer mechanism in slices. However, the finding, in turn, might suggest that hydrolysis of labeled protein, *i.e.*, the last step in hormone synthesis in thyroid, is really occurring in slices.

With regard to effect of TSH, it was observed that each phase of iodine metabolism in thyroid slices was, more or less, affected by the addition of TSH to the incubation medium. Among these effects, most interesting is the one upon the conversion of iodide to protein-bound iodine.

Here, it should be remembered that specific radioactivities of iodide both in slices and in medium varied with time during incubation (Fig. 10). Accordingly, the observed change in the rate of I^{131} incorporation into protein fraction of thyroid caused by TSH does not *a priori* mean the change in the rate of conversion of iodide to protein-bound iodine.

Therefore, calculation of true conversion rates of iodine seems to be valuable. If it is permitted to assume that all of iodide ions in slices are biochemically equivalent, rates of iodine transfer can be calculated from the observed data using the following equations (the derivation of these equations as well as assumptions employed are described in

Appendix).

$$R_{in}=\frac{(I_s)_{t_2}-(I_s)_{t_1}+\frac{(I^{o}_{m})_{t_2}-(I^{o}_{m})_{t_1}}{t_2-t_1}\int_{t_1}^{t_2}A_s\,dt}{\int_{t_1}^{t_2}A_m\,dt-\int_{t_1}^{t_2}A_s\,dt} \qquad \text{(I)}$$

$$R_{out}=R_{in}+\frac{(I^{o}_{m})_{t_2}(I-{}^{o}_{m})_{t_1}}{t_2-t_1} \qquad \text{(II)}$$

$$R_p=\frac{(I_p)_{t_2}-(I_p)_{t_1}}{\int_{t_1}^{t_2}A_s\,dt} \qquad \text{(III)}$$

Here, R_{in}, R_{out}, and R_p are the rate of iodide transfer from medium to slices, the rate of iodide transfer from slices to medium and the conversion rate of intrathyroidal iodide to protein-bound iodine, respectively. I_p and I_s represent radioactivity of intrathyroidal protein and total activity in slices, respectively, and I^{o}_{m} means total amount of iodide in medium. Specific radioactivities of iodide in medium and in slices are shown by symbols A_m and A_s, respectively. t_1 and t_2 are the time of observation and the symbols $(I_s)_{t_1}$, $(I_s)_{t_2}$, etc. represent the values I_s or others obtained at time t_1 or t_2.

TABLE III

Calculated Rates of Iodine Transfer in Thyroid Slices

Expt. No.	Phase of incubation		Rates of iodine transfer (mμg./min./100 mg. tissue)					
			R_p		R_{in}		R_{out}	
			Control	TSH	Control	TSH	Control	TSH
	min. —	min.						
1	0	5	2.4	2.6	3.5	3.0	6.6	6.9
	5	20	0.6	1.2	4.0	4.2	6.6	4.0
	20	40	0.2	0.5	3.7	4.0	4.0	4.0
	min. —	min.						
2	10	40	6.8	47	3.4	3.8	5.1	6.2
	20	40	22	25	3.1	3.0	3.3	3.3

R_{in} is rate of iodide transfer from medium to slices, R_{out} is rate of iodide release from slices to medium and R_p is rate of iodide transfer from iodide pool to protein-bound iodine. These values were calculated from equations (I)—(III).

Table III shows the calculated values of R_{in}, R_{out} and R_p in the presence or absence of TSH in the incubation medium. It can be seen that TSH effect is only distinct in the rate of intrathyroidal conversion of iodide to protein-bound iodine, R_p, early in the incubation. The reason for the decrease of the response elicited by TSH in later period of incubation (in Experiment 2) is not clear. It might be due to destruction of TSH-sensitive mechanism during prolonged incubation.

In studies on the action of TSH in thyroid slices, most of other investigators have only observed the change in total I^{131} incorporation after a prolonged incubation (2-4 hours) with TSH (*2–4*, *6*). Even in other investigation (*5*) in which incubation time was 15 minutes, TSH effect was studied only in total uptake of I^{131}. It is evident, from the results of the present investigation, that these experiments provide inconsistent results which reflect complicated intrathyroidal iodine metabolism and susceptibility of thyroid slices to incubation.

From this point of view, the finding in the present study that TSH definitely stimulates the conversion of intrathyroidal iodide to thyroglobulin-bound iodine early in the incubation period seems to be important. It is also to be noted that this effect is one of the earliest *in vitro* effects previously reported. Therefore, more extended study on this effect might be useful for the elucidation of the mechanism of TSH action.

SUMMARY

Making use of pig thyroid slices, various phases of iodine metabolism and effects of

thyrotropic hormone (TSH) upon them were studied, and the following results were obtained.

1. Intrathyroidal iodide pool size does not change during incubation in spite of the presence of active iodide transport and release of iodide.

2. Conversion of iodide to protein-bound iodine was found to occur slowly, but lasted for rather long time. Newly formed protein-bound iodine exists as monoiodo- and diiodotyrosines in thyroglobulin molecules.

3. Labeled organic iodine, *i.e.*, monoiodo- and diiodotyrosines, iodinated lipid and thyronine-like substances, are released from slices after prolonged incubation.

4. During initial period of incubation, TSH definitely stimulates the conversion of intrathyroidal iodide to thyroglobulin-bound iodine without affecting the total iodine uptake. The effect decreases with duration of incubation.

5. The change in specific radioactivity of iodine in slices and medium as well as conversion rates of iodine transfer were calculated in the presence and absence of TSH.

The author wishes to express his gratitude to Prof. N. Ui for his guidance in the course of the work. He is also indebted to Dr. M. Nomoto, Institute of Physical and Chemical Research, for the gift of "Pronase" and to Taiyō Gyogyō Kaisha Ltd. for the supply of whale pituitaries. The expense of this study was defrayed in part by a grant from the Ministry of Education.

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APPENDIX

Equations (I) to (III) described in Discussion were derived as follows.

If it is assumed that the specific radioactivity of iodide is same all over the gland, *i.e.*, thyroid has a single iodide pool, radioactivity accumulated in slices between times t_1 and t_2 is given by

$$(I_s)_{t_2}-(I_s)_{t_1}=\int_{t_1}^{t_2} R_{in}\ A_m dt-\int_{t_1}^{t_2} R_{out}\ A_s dt$$

Here, I_s is the total radioactivity in slices and R_{in} and R_{out} are the rates of iodide transfer from medium to slices and from slices to medium, respectively. A_m and A_s mean specific activities of iodide in medium and in slices, respectively.

On the other hand, the change in total iodide content in medium is represented by the equation

$$(I_m^o)_{t_2}-(I_m^o)_{t_1}=\int_{t_1}^{t_2} R_{out}\ dt-\int_{t_1}^{t_2} R_{in}\ dt$$

If it is assumed that R_{in} and R_{out} are constant from t_1 to t_2, these two equations can be rearranged to

$$(I_s)_{t_2}-(I_s)_{t_1}=R_{in}\int_{t_1}^{t_2}A_m\,dt-R_{out}\int_{t_1}^{t_2}A_s\,dt$$

and

$$(I_m^o)_{t_2}-(I_m^o)_{t_1}=(R_{out}-R_{in})\ (t_2-t_1)$$

Hence, the following equations are easily obtained:

$$R_{in}=\frac{(I_s)_{t_2}-(I_s)_{t_1}+\dfrac{(I_m^o)_{t_2}-(I_m^o)_{t_1}}{t_2-t_1}\displaystyle\int_{t_1}^{t_2}A_s\,dt}{\displaystyle\int_{t_1}^{t_2}A_m\,dt-\int_{t_1}^{t_2}A_s\,dt}\qquad\text{(I)}$$

$$R_{out}=R_{in}+\frac{(I_m^o)_{t_2}-(I_m^o)_{t_1}}{t_2-t_1}\qquad\text{(II)}$$

In the same manner, if the rates of iodine conversion are constant from t_1 to t_2, accumulation of radioactivity of protein-bound iodine from t_1 to t_2 is given by

$$(I_p)_{t_2}-(I_p)_{t_1}=R_p\int_{t_1}^{t_2}A_s\,dt-R_h\int_{t_1}^{t_2}A_p\,dt$$

Where R_p and R_h are rates of conversion of iodide to protein-bound iodine and conversion of protein-bound iodine to iodide or iodinated amino acids, respectively, and A_p is the specific activity of protein-bound iodine. If it is taken into consideration, that the specific activity of protein-bound iodine is much smaller than that of iodide at least at the initial period of incubation, second term of this equation can be neglected and the equation is simplified as

$$R_p=\frac{(I_p)_{t_2}-(I_p)_{t_1}}{\displaystyle\int_{t_1}^{t_2}A_s\,dt}\qquad\text{(III)}$$

The Journal of Biochemistry, Vol. 50, No. 2, 1961

Effect of Thyrotropic Hormone on the Iodination of Thyroglobulin in Pig Thyroid Slices

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(Received for publication, March 13, 1961)

In the previous study (*1*), incorporation of I^{131} into pig thyroid slices and its conversion to protein-bound iodine were investigated in the presence or absence of thyrotropic hormone (TSH). As a result of this study, it has been found that, during the initial incubation period (the first 10 to 30 minutes), TSH does not cause an appreciable change in the total I^{131} uptake by the slices, as well as in the intracellular pool size of iodide, but definitely enhances the labeling of protein fraction in the slices. On the other hand, when the incubation is prolonged for more than an hour, the stimulation of the labeling of the protein by TSH diminishes and occasionally inhibition occurs according to the state of thyroid gland. Therefore, it is evident that more extended observation of I^{131} incorporation during the initial period of incubation is valuable as the first step of studies on the effect of TSH upon thyroid glands.

In the present paper, the results of further studies about the early effect of TSH on the incorporation of I^{131} into the protein of thyroid slices are described.

EXPERIMENTALS

TSH—The following three preparations were used. Whale thyrotropic hormone was prepared according to the method described previously (*1*) (*ca.* 1 U.S.P. unit/mg.). Thyrotropin, U.S.P. Reference Standard was obtained from American Roland Corp., New York and its biological activity was 0.074 U.S.P. units/tablet (*ca.* 20 mg.). Pretiron, commercial product of Shering A.G., Berlin, was also used (*ca.* 9 J.S. units/mg.).

Method—Pig thyroid slices (100–200 mg.) were incubated, unless otherwise stated, in the standard incubation medium (3 ml.) described in the previous paper (*1*), *i.e.*, the medium containing KI, NaI^{131} and Krebs-Ringer phosphate solution (pH 7.2). Other procedures including I^{131} and I^{127} analyses, paper electrophoresis, and paper chromatography were described in the previous paper (*1*).

RESULTS

Fate of I^{131} in Slices and TSH Effect—In order to obtain further information concerning the effect of TSH which is observed during earier period in the course of labeling of protein fraction in thyroid slices, the following experiments were performed using thyroid slices obtained from the same animal.

In Experiment 1, thyroid slices were incubated with labeled iodide for 6 minutes and then the labeled slices were re-incubated for 12 minutes in a non-labeled medium containing I^{127}-iodide as usual. The radioactivities of ethanol-soluble and -insoluble fractions in the slices were determined before and after the second incubation. In Experiment 2, the first incubation was performed in the absence of radioactive iodide while the second in the presence of I^{131}. Distribution of I^{131} in the slices was determined after the second incubation. In both experiments, TSH was added to the medium in the second incubation and the results were compared with those obtained when TSH was omitted. The results are summarized in Table I.

A fairly large part of iodide in slices was found to be released into the medium by the incubation (Experiment 1, second incubation), while an active incorporation of iodide continued at the same period (Experiment 2, second incubation). However, TSH altered neither the extent of the release of iodide nor the

TABEE I

Fate of I^{131}-Iodide in Slices during Incubation in the Presence or Absence of TSH

I^{131} incorporated (c. p. m. × 10^{-2})

Expt. No.	After the first incubation1)		After the second incubation2)				Release from slices3)	
	Total	Protein-bound	Total		Protein-bound			
			Control	TSH	Control	TSH	Control	TSH
1	575	122	274	285	111	158	301	290
2	—	—	736	743	153	214	—	—

1) Slices were incubated either with I^{131}-labeled KI (Expt. 1) or with non-labeled KI (Expt. 2) for 6 minutes at 37°C.

2) After the first incubation, slices were re-incubated either with non-labeled KI (Expt. 1) or with I^{131}-labeled KI (Expt. 2) for 12 minutes at 37°C.

3) Total I^{131} in slices after the first incubation minus total I^{131} in slices after the second incubation.

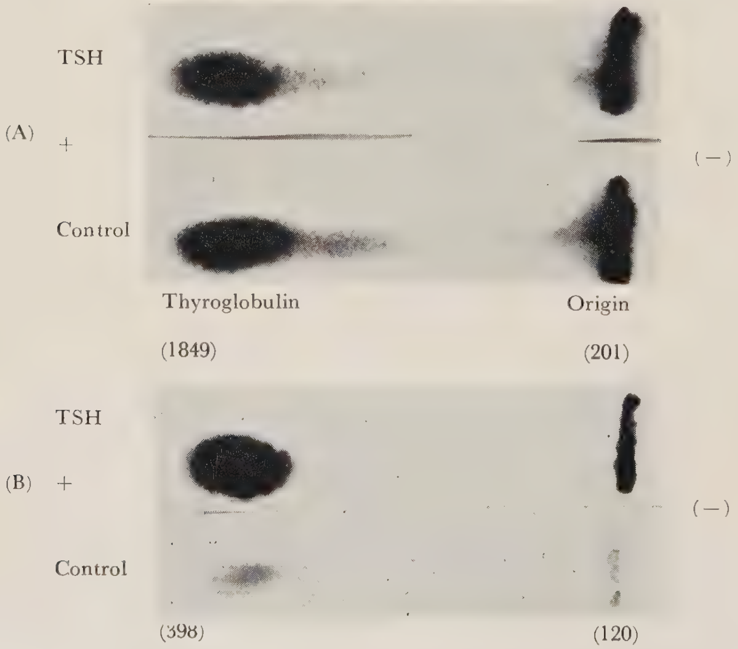

FIG. 1. Electrophoretic diagram of proteins in thyroid slices after the incubation of the slices with NaI^{131} in the presence or absence of TSH.

Electrophoresis: in a veronal buffer (pH 8.6, $\mu=0.075$); constant current density, 0.45 mA/cm.

(A) Paper electrophoretic diagram stained with BPB.

(B) Radioautogram

Numerical values enclosed in bracket express radio-activities of the areas in c. p. m.

extent of the iodide transfer from the medium to the slices. The effect of TSH only observed was the increase in the radioactivity of ethanol-insoluble fraction at the expense of iodide in slices. Therefore, TSH was thought, under the present condition, to stimulate the process of conversion of intrathyroidal iodide to protein-bound iodine exclusively.

Identification of TSH-Sensitive Protein—In order to determine the nature of the protein into which TSH-dependent incorporation occurred, homogenates prepared from the thyroid slices which had been incubated with labeled iodide in the presence or absence of TSH were examined by means of paper electrophoresis.

In Fig. 1 are shown the typical radioautograms of the electrophoretic strips thus obtained. The values of radioactivities of the components appeared on the paper are also included in this figure. The substantial difference was included in the radioactivity of one component migrated toward the anode. This component was identified as thyroglobulin from its behavior in the electric field.

Paper chromatographic examination of enzymatic digest showed that a large part of I^{131} in the protein fraction existed as monoiodotyrosine and diiodotyrosine.

From these resuls, it was concluded that the enhanced incorporation caused by the added TSH was due to enhanced iodination of thyroglobulin, as it occurs *in vivo*.

Specificity of TSH as Stimulator—Three different preparations of TSH showed essentially the same stimulatory effect upon the incorporation of I^{131} into the protein fraction. On the contrary, when ACTH (commercial product of N. V. Organon, Netherland) or serum albumin was added to the incubation medium instead of TSH at the concentration of 33 μg. per ml., no observable change was brought about. Thus, specificity of TSH in this reaction was suggested. The results are shown in Table II.

Dose-Response Curve—When the concentration of TSH in the incubation medium was varied from 0.3 μg. to 33 μg. per ml., alinear increase in the radioactivity of the protein

TABLE II

Specificity of TSH as Stimulator

Addition[1)]	I^{131} uptake	
	Total uptake in slices (c.p.m. × 10^{-3})	% of uptaken in protein
None	137	16.3
Serum albumin	179	15.1
ACTH	161	18.2
TSH[2)]	175	42.9
TSH[3)]	189	44.6
None	125	16.3
TSH[2)]	165	57.2
TSH[4)]	188	49.2

1) Concentration: 33μg./ml. except in the case of TSH[4)].

2) Whale thyrotropic hormone

3) "Pretiron"

4) Thyrotropin U. S. P. Reference Standard

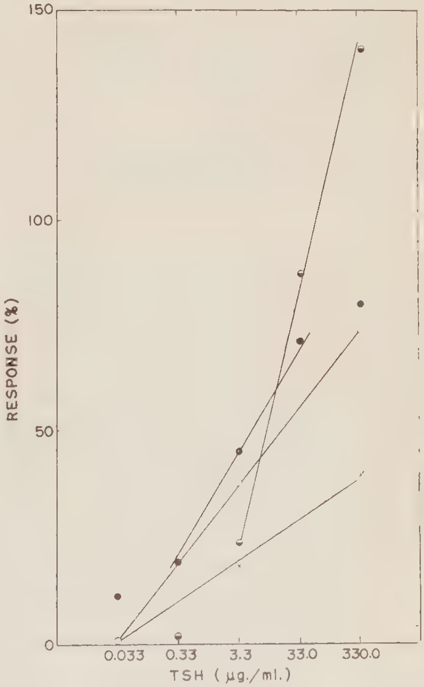

FIG. 2. Dose-response curve of different thyroid slices.

Concentration of TSH is expressed as logarithm of μg./ml. in abscissa. Incubation: 15 minutes at 37°C.

TABLE III

Effect of Various Reagents on I^{131} Uptake by Thyroid Slices and on TSH Action

Reagents tested	Total uptake (relative radioactivity[1)])		Protein-I^{131} (% of I^{131} recovered in protein fraction)	
	Control	TSH	Control	TSH
No addition	100	83.4	10.0	25.0
1×10^{-5} *M* DNP	98.6	69.5	8.5	29.2
1×10^{-4} *M* DNP	83.1	82.3	6.6	10.9
1×10^{-3} *M* EDTA	92.5	65.4	9.0	28.8
1×10^{-2} *M* EDTA	97.5	93.4	7.0	11.7
No addition	100	81.5	24.9	37.0
3×10^{-3} *M* NaF	94.5	93.8	26.7	39.0
3×10^{-5} *M* PCMB	107	84.2	23.0	31.1
3×10^{-4} *M* KCN	96.9	84.0	19.3	28.5
3×10^{-3} *M* KCN	66.9	74.1	10.8	15.4
No addition	100	92.0	10.0	17.1
1×10^{-3} *M* Cysteine	97.0	66.3	10.0	10.9
No addition	100	109	12.9	21.2
1×10^{-3} *M* Methylmercaptoimidazole	83.5	83.5	9.1	6.7
1×10^{-3}*M* Dithiodiethylcarbamate	114	90.0	6.6	7.0
2×10^{-3} *M* KSCN	25.6	23.0	8.6	8.6
2×10^{-3} *M* Ascorbic acid	98.5	93.0	11.7	12.8

1) Taken the total I^{131} uptake in the case when TSH and other reagents were not added as 100.

fraction was observed with the increase in the logarithm of the TSH concentration. Four does-response curves obtained with thyroid slices of different pigs are shown in Fig. 2. The slope of dose-response curve was found to vary considerably with slice preparations. However, it is to be noted that neither horizontal nor negative (inhibitory) dose-response curve has been obtained yet. This is an important feature of this reaction, since, when the incubation was continued for more than an hour instead of nearly 15 minutes' incubation in the present study, the response elicited by TSH was found to be either stimulatory, no effect or inhibitory according to the state of thyroid glands (*1*).

Effect of the Addition of Various Reagents—By the addition of various reagents to the medium, different effects were observed upon the I^{131} incorporation. The results are summarized in Table III.

The total uptake of I^{131} was not influenced in every case except in the case of thiocyanate and high concentration of cyanide. Other anti-thyroid compounds inhibited the conversion of iodide to protein-bound iodine even in the presence of TSH. It is of value to note that cysteine and ascorbic acid which acted possibly as reducing agents strongly inhibited the action of TSH without any influence on the I^{131} transfer in the absence of TSH. Cyanide, relatively high concentration of dinitrophenol (10^{-4}*M*) as well as 10^{-2} *M* ethylenediaminetetraacetate inhibited the conversion of iodide to protein-bound iodine rather than the total I^{131} uptake by the slices. This inhibition recovered partially in the presence of TSH.

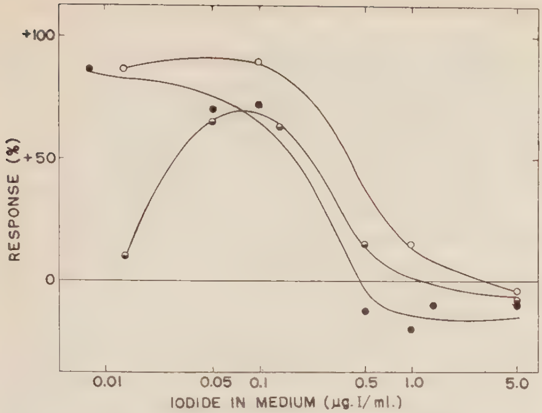

Fig. 3. Effect of iodide concentration in medium upon TSH action.

Thyroid slices were incubated with NaI^{131} in the presence or absence of TSH. Incubation: 15 minutes, at 37°C. Figure shows the results of three experiments using different thyroid glands.

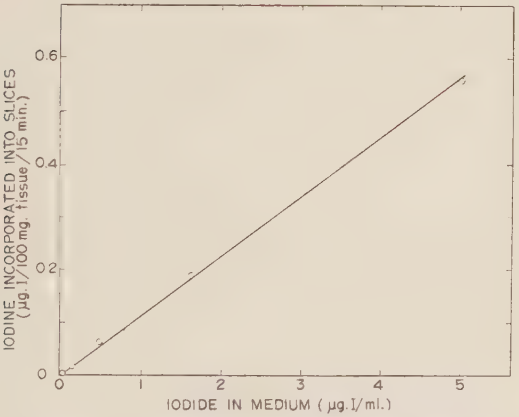

Fig. 4. Effect of concentration of iodide in medium upon the rate of iodide transport to slices.

Effect of Concentration of Iodide in the Medium—The effect of iodide concentration in the medium upon the TSH action is shown in Fig. 3. Linear increase in the total amount of iodine incorporated into slices was found with increase in the concentration of iodide in the medium up to 5 μg./ml. (Fig. 4). Neverthless, the addition of TSH did not alter the labeling of protein fraction in thyroid slices if the iodide concentration was higher than 0.5–1.0 μg./ml. (Fig. 3). It seems interesting that this concentration is approximately equal to the concentration of iodide in thyroid slices.

DISCUSSION

Many authors have reported the effect of TSH *in vitro* on the iodine transfer (*2–8*), P^{32} uptake (*9*), wet weight of slices (*10*), and on glucose oxidation (*11*). Some of them have been applied as assay methods of TSH (*2*, *5–10*). In most of the previous experiments, incubation of slices was performed for more than 2 hours. As previously described (*1*), the prolonged incubation brings about complication in the iodine metabolism in survival tissue slices. The system described in this paper excludes such a confusion, since what is observed is mainly the action of TSH on the process of conversion of iodide to protein-bound iodine. Neverthless, the relatively gentle slope of the dose-response curve and its variation with different slice preparations might indicate the complex nature of the action of TSH.

This stimulatory effect on the incorporation would be usable as an assay method of TSH. However, it might be necessary for the purpose of obtaining accurate results to use mixed slices obtained from a number of glands and average responses to TSH or to select every time the gland with high responsibility.

The effect of iodide concentration upon TSH action seems to be interesting. Under physiological condition in which concentrations of substrates are not in saturation, the conversion of iodide to thyroglobulin-bound iodine is stimulated by TSH. On the contrary, when the concentration of iodide (one of the substrates) becomes sufficiently high, though not physiological, any enhancement of iodide conversion by TSH does not occur, while the rate of conversion itself becomes higher.

SUMMARY

1. Thyrotropic hormone (TSH) enhanced the process of the conversion of iodide to protein-bound iodine when pig thyroid slices were incubated with TSH for a short period.

It was proved that TSH-sensitive reaction was iodination of thyroglobulin.

2. Specificity of this reaction was confirmed since three different preparations showed the same effect and serum albumin or ACTH had no effect.

3. The extent of the conversion of iodide to protein-bound iodine was proportional to the logarithm of TSH concentration, though the slope of the dose-response curve considerably varied with glands from which slices were prepared.

4. Anti-thyroid compounds and other reagents affected the iodine transfer and the TSH effect in various manners.

The author wishes to express his sincere thanks to Prof. N. U i for his guidance and encouragement during this work. The expense of this study was defrayed in part by a grant from the Ministry of Education.

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The Journal of Biochemistry, Vol. 50, No. 2, 1961

The Difference between Normal Liver, and Regenerating Liver and Hepatomas of Rat in an Enzymatic Reaction Forming Ethanolamine*

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(Received for publication, March 13, 1961)

While establishing a method for the microdetermination of tissue amines, we found that a much larger amount of ethanolamine is detectable under certain conditions in hepatomas and regenerating liver than in normal liver of rat. This was confirmed in regenerating liver by determining the amount of ethanolamine and the mitotic rate during regeneration.

The present paper reports these results and describes the identification of ethanolamine. The preliminary observation that the ethanolamine detected is a product of an enzymatic process during the determination is also reported.

MATERIALS AND METHODS

Rats—Male Sprague-Dauley strain rats weighing approximately 200 g. were used, except when otherwise stated. Before use they were kept in an air-conditioned room (22±2°C) for at least two days with normal food and water.

Hepatomas — *p*-Dimethylaminoazobenzene (DAB)-induced hepatomas of rats were obtained by feeding a semi-synthetic diet containing 0.08% DAB for about 5 months. AH-130 ascites hepatomas were harvested 8 to 10 days after transplantation and washed with isotonic saline.

Regenerating Liver—Rats were partially hepatectomized under ether anesthesia, by the method of Higgins and Anderson (*1*). About two thirds of the liver was removed. After the operation, food and water were given without restriction.

Estimation of Tissue Amines—Except when otherwise stated, the tissue was homogenized in about ten volumes of 50 per cent aqueous acetone. If homogenates had been made in distilled water and subjected to some treatment, such as heat treatment to stop the enzymatic reactions, an equal volume of acetone was added. To 20 ml. of the aqueous acetone homogenate 0.1 ml. of 1-fluoro-2, 4-dinitrobenzene (FDNB) and 2 g. of sodium bicarbonate were added. The suspension was stirred vigorously with a magnetic stirrer for 3 hours at room temperature, and then about 0.1 g. of glycine was added and the stirring continued for a further hour to remove excess FDNB which subsequently interfered with the extraction of dinitrophenyl (DNP)-amines. The resulting suspension was then centrifuged, and the supernatant and the precipitate were extracted separately with ether. After washing with 2% sodium bicarbonate, the two ether extracts were combined and concentrated. The resulting amorphous and sometimes oily material was chromatographed on a column of Amberlite IRC-50 (H^+form, 200~300 mesh) by the method of Seki and Morimoto (*2*), using a mixture of methyl-ethyl-ketone, tetrahydrofuran and water (3 : 4 : 13) as solvent. Except during preparation of the sample used for identification of the amines, commercially available solvents were used without further purification. The eluate from the column (0.9×50 cm.) was collected in 1 ml. fractions, and the optical density of each was determined at 360 mμ after the addition of 3 ml. of a mixture of 1 per cent HCl and acetone (3 : 2). 0.1 to 1.5 μmoles of DNP-ethanolamine was a suitable amount for application to the column.

The elution pattern of DNP-amines is shown in Fig. 1. The ratio of the tube number of the peak for DNP-ethanolamine to that for dinitroaniline was 0.74±0.01 and was a good measure for the identification of DNP-ethanolamine.

The recovery of amines during the whole procedure was determined by tests with known amounts of amines added to tissue homogenates and the isolated amines were determined after the treatment described above. The results are presented in Table I. Aliphatic mono-amines, especially ethanolamine, were recovered in good yield.

The molecular extinction coefficients and some chemical properties of DNP-amines are presented in the APPENDIX.

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* A part of this work was presented at the 33rd Congress of the Japanese Biochemical Society held in Tokyo in November, 1960.

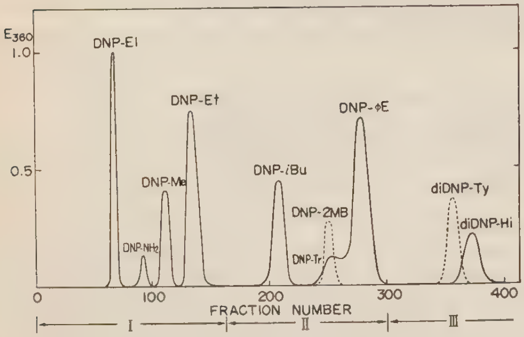

Fig. 1. Chromatogram of authentic DNP-amines. An Amberlite IRC-50 column (H^+form, 200~300 mesh, 0.9×50 cm.) was used. The solvents used were methyl-ethyl-ketone (MEK)~tetrahydrofuran (THF)~water (3 : 4 : 13) in I; MEK-THF-water (3 : 4 : 12) in II; and MEK-THF-water (3 : 4 : 10) in III. The following abbreviations are used: El, ethanolamine; DNP-NH_2, dinitroaniline; Me, methylamine; Et, ethylamine; *i*Bu, *iso*butylamine; Tr, Tryptamine; 2MB, 2-methyl-butylamine; φE, phenethylamine; Ty, tyramine; Hi, histamine. The colorimetry method is described in the text.

Table I

Recovery of Amines after Dinitrophenylation of the Tissue Homogenate, Extraction and Chromatography

Amines	Recovery (%)	Recovery after Chromatography (%)[1)]
Ethanolamine	96, 103	103, 109, 99
Methylamine	101	99, 90, 91
Ethylamine	90	105, 97, 98
*iso*Butylamine	99, 88, 87	107, 101, 95
Phenethylamine	96, 81	87, 91, 81
Tryptamine	35	90
Tyramine	26	91, 65
Histamine	56	93

The calculation was as follows.

Recovery = {(amine + homogenate) − (homogenate)} × 100/(amine added). The values in parentheses are for amines found after the procedure. Substraction of (homogenate) was neglected except with ethanolamine.

1) Recovery of authentic DNP-amines after column chromatography.

RESULTS

The Ethanolamine Content of Tissues Estimated without Heat Treatment—The elution patterns shown in Fig. 2 are typical examples of chromatograms of ether extracts of dinitrophenylated tissue homogenates of normal liver and DAB-hepatomas of rat. The homogenates were not heated in the course of experiments to stop enzymatic reactions. As can be seen, a considerable amount of a DNP-derivative, which coincided in position with DNP-ethanolamine, was detected in DAB-hepatomas, though there was very little of it in normal liver. The identification of this DNP-derivative as DNP-ethanolamine will be described in a later section.

Ethanolamine was estimated in DAB-hepatomas, AH-130 ascites hepatomas, regenerating liver obtained 37~48 hours after partial hepatectomy, and normal livers of rats. The results in Table II shows that the ethanolamine contents of hepatomas and regenerating liver are much larger than that of normal liver.

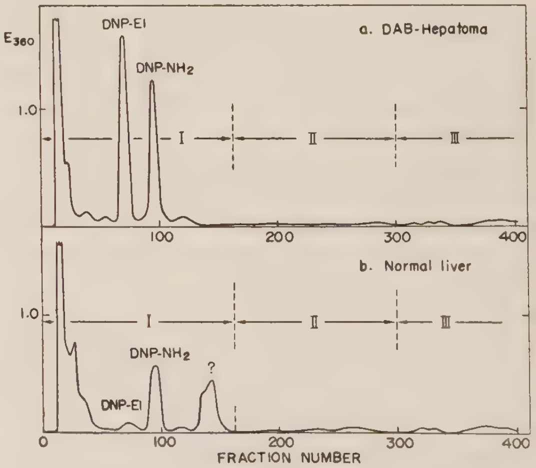

Fig. 2. Chromatograms of ether extracts of dinitrophenylated homogenates of a DAB-hepatoma (a), and normal liver (b) of rat. Samples (a) and (b) applied to the column corresponded to 1.1 g. and 1.4 g. of the original tissues, respectively. For abbreviations used, see Fig. 1. (The "?"-marked peak was colorless.)

TABLE II

Ethanolamine Content of Various Tissues of Rat

Tissue	Ethanolamine found			
	μmoles/g. wet tissue		μmoles/g. total N[1]	
Normal liver	0.11, 0.09 0.40, 0.48 0.09, 0.23 0.26, 0.20 0.12	0.22 ±0.13[2]	12, 17 4, 7 8, 6 4	8±3[2]
Regenerating liver (37~48 hours)	2.18, 1.99 1.31, 1.47 1.30, 1.92 2.47, 1.58 2.06, 1.81	1.81 ±0.37[2]	72, 46 55, 48 67, 81 49, 52 74	60±12[2]
DAB-hepatoma	1.86 (1.81)[3] 1.57 (0.27)[4]		90	
AH-130 ascites hepatoma			91 91	

For conditions, see text.

1) Total N was determined by the micro-Kjeldahl method on an aliquot of homogenate.
2) Mean value and standard deviation.
3) Necrotic part of the same tissue.
4) Normal part of the liver.

Ethanolamine Content and Mitotic Rate during Regeneration of Liver—Male Donryu-rats* of about 200 g. body weight were used in this experiment. The ethanolamine content and mitotic rate were determined in the course of liver regeneration, using two rats each time. A small part of liver cut off from a definite region was used for counting the mitotic number. The remaining major portion was used for ethanolamine estimation without heating, as for the work described in the above section. As shown in Fig. 3, the ethanolamine content increased rapidly until 2 days after partial hepatectomy, and at this time the mitotic rate was also maximal. After this period, the content of ethanolamine decreased gradually to the normal level. As a control for the operation, laparotomized rats were used, and they showed normal ethanolamine values.

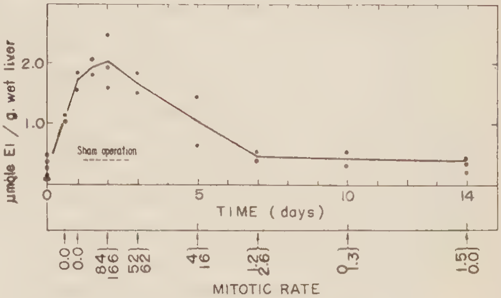

FIG. 3. Time course of the change in ethanolamine content and the mitotic rate during liver regeneration. The level indicated as "sham operation" is the value 48 hours after mere laparotomy. Mitotic rates are shown as the number of metaphase nuclei found in 100 microscopic fields at ×400 magnification.

Identification of Ethanolamine—Chromatographic eluates of the DNP-amine, regarded as DNP-ethanolamine in the above sections, from

TABLE III

Identification of the Unknown DNP-derivative

	The unknown derivative	DNP-ethanolamine
1. Melting point[1]	88.5~89°C	88~89°C
	Mixed sample 88~89°C	
2. Optical characteristics of solutions		
a. In 1% HCl~acetone (3:2)		
Absorption maximum	357 mμ	357 mμ
E_{357}/E_{410}	2.36	2.39
b. In dioxane		
Absorption maximum	346 mμ	346 mμ
E_{346}/E_{400}	2.96	2.93
3. Paper chromatography		
a. Methyl-ethyl-ketone~tetrahydrofuran~water (1:1:2)		
R_f	0.74	0.74
b. Benzene~water (1:1)		
R_f	0.78	0.77

1) Uncorrected.

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* "Donryu-rat" is the name of a colony of Japanese rats which have proliferated from a single pair by random breeding since 1950 (*3*).

many samples of regenerating liver, were pooled and purified twice more by the same chromatographic treatment. For the final chromatography the solvents were redistilled before use. By these treatments colorless oily contaminants were removed. The final DNP-ethanolamine fraction was dried, and the resulting crystalline material was dissolved in a small amount of ethanol. From this solution a DNP-derivative was recrystallized by dropwise addition of water. Its melting point was identical with that of an authentic sample of DNP-ethanolamine. Moreover, the absorption spectrum and paper chromatographic behavior of the unknown and authentic samples in two different solvent systems were the same (Table III).

Enzymatic Study of the Appearance of Free Ethanolamine—In all the experiments described above, the tissue homogenates in 50% aqueous acetone were dinitrophenylated directly without stopping enzymatic processes.

When a freshly prepared aqueous homogenate of regenerating liver was treated with trichloroacetic acid (TCA) or heated at 100°C for 5 minutes, its ethanolamine content was reduced to about 12 per cent of that obtained in untreated samples (Table IV).

Thus most of the ethanolamine detected is probably a product of some enzymatic reaction occurring during dinitrophenylation in 50% aqueous acetone. To obtain further information on this, the following experiment was carried out: Liver homogenates in distilled water were incubated at 37°C and aliquots

Table IV

Effect of Various Treatments on the Ethanolamine Found

Treatment	Ethanolamine found
Control	1.29 µmoles/g. wet liver
TCA	0.16
	0.16 ......supernatant
	0.004......precipitate
	0.000......ether extract
Heating at 100°C for 5 minutes	0.14

An equal volume of 12% TCA was added to an aqueous homogenate of regenerating liver. After centrifugation the supernatant was extracted with ether containing a few drops of conc. HCl to remove TCA. Fractions were dinitrophenylated separately. The heat treated homogenate was then immediately dinitrophenylated as usual.

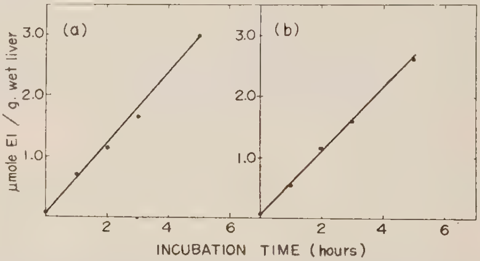

Fig. 4. Effect of incubation time on the ethanolamine content. Aqueous homogenates of normal liver (a) and regenerating liver (b) were incubated at 37°C. For procedures, see text.

Table V

Effect of Acetone on the Rate of Ethanolamine Production

		Regenerating liver		Normal liver	
	Time after operation (hrs.)	Homogenate conc. (g. liver/ml.)	Rate of ethanolamine production (µmole/g. liver/hr.)	Homogenate conc. (g. liver/ml.)	Rate of ethanolamine production (µmole/g. liver/hr.)
—Acetone	72	0.15	0.51	0.27	0.57
	45	0.14	0.48	0.145	0.61
+Acetone	45	0.14	0.37[1]	0.145	≈0.01

Homogenates were incubated with and without an equal volume of acetone at 37°C.

1) In this case the rate was not constant, and the increase during the first one hour is shown.

were withdrawn at appropriate intervals. After heating each aliquot at 100°C for 5 minutes, the amount of ethanolamine was estimated under the conditions described above. The results for normal and regenerating liver are shown in Fig. 4. The ethanolamine content increased linearly for at least 5 hours, and the homogenate at zero time contained scarcely any amine. Furthermore, contrary to expectation, the rate of ethanolamine production in a normal liver homogenate was the same as that in a homogenate of regenerating liver.

The Cause of the Difference in Ethanolamine Content of Normal and Regenerating Liver—When homogenates were incubated in 50% acetone, there was a difference in the ethanolamine content of normal and regenerating liver. Table V shows that the production of free ethanolamine is almost completely suppressed in a normal liver homogenate, while in a homogenate of regenerating liver there is only about 30% suppression in the initial velocity.

DISCUSSION

The amount of free ethanolamine in homogenized animal tissues can be quantitatively estimated as follows: dinitrophenylation of homogenized tissue, ether extraction of DNP-derivatives, column chromatography on Amberlite IRC-50 resin using a mixture of methyl-ethyl-ketone, tetrahydrofuran and water as solvent, and colorimetry. This method is applicable to usual aliphatic monoamines. It can also be used for other kinds of amines, with improved extracting and eluting solvents. It should be emphasized that tissues usually contain enzymes causing the formation of ethanolamine during dinitrophenylation, and that, to estimate the true *in vivo* content, an adequate method for inactivation of these enzymes, as for example, heat treatment at 100°C for 5 minutes, is necessary immediately after homogenization.

It has been concluded that mammalian tissues contain very little or no free ethanolamine (4, 5). In the present experiments both regenerting and normal rat liver were found to contain less than 0.05 μmole per g. wet liver *in vivo*. However, an enzyme or enzyme system producing free ethanolamine (for example, some phosphatases) seems to be present in rat liver, and after homoegnization ethanolamine is formed at a considerable rate (Fig. 4). More detailed enzymatic studies on this phenomenon are needed.

During incubation at 37°C, aqueous homogenates of both normal and regenerating liver produce free ethanolamine in a similar way (Fig. 4). But when acetone is present in the homogenates, the reaction proceeds in quite a different way in the two (Table V). In homogenates of regenerating liver free ethanolamine increases at a velocity of about two thirds of that when acetone is absent, while in normal liver homogenates there is very little free ethanolamine and its level remains practically constant during a 3 hour incubation period. Tumor homogenates are similar to those of regenerating liver in this respect.* The cause of the difference between normal liver and the two other homogenates in acetone is the second point to be studied. The apparent difference in their ethanolamine contents (Table II) is probably due to this phenomenon.

It is easily conceivable that rapidly growing tissues, such as regenerating liver or tumor, have the ability to synthesize all cell constituents rapidly. The parallelism in liver regeneration between the rate of ethanolamine production in aqueous acetone homogenates (presented as the "content" in Fig. 3) and the mitotic rate (Fig. 3), suggests that there is some relationship between ethanolamine production and growth. The second point mentioned in the above paragraph is very interesting in this connection.

The nature of the enzyme(s) concerned and the effect of acetone are under investigation.

SUMMARY

1. A method for the determination of amines in tissues is described.

2. The apparent content of ethanolamine of rat hepatoma and rat regenerating liver

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* Unpublished data.

was about 8 times greater than that in normal liver.

3. The ethanolamine content increased in parallel with the mitotic rate during liver regeneration.

4. The difference in the ethanolamine content of these tissues was attributed to an enzymatic reaction which produced free ethanolamine. Addition of an equal volume of acetone to a tissue homogenate almost completely inhibited this reaction in normal liver, while inhibition by acetone was small in homogenates of regenerating liver.

The authors wish to thank Prof. M. Kuru, the Director of this Institute, and Prof. S. Akabori of the Institute for Protein Research, Osaka University, for their continuous encouragement and interest in this work, and Drs. T. Fukui and S. Morishita for their co-operation in histological work. Thanks are also due to Misses T. Miyaji and C. Iwasaki for their skilled technical assistances.

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The Journal of Biochemistry, Vol. 50, No. 2, 1961

APPENDIX

Some Properties of DNP-Amines*

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(Received for publication, March 13, 1961)

Amines were dinitrophenylated by 1-fluoro-2, 4-dinitrobenzene in 67% aqueous acetone containing an appropriate amount of sodium carbonate, except for tyramine, spermidine and spermine, which were dinitrophenylated in the absence of acetone. DNP-2-methylbutylamine was kindly given by Dr. T. Seki of the Department of Genetics, Osaka University Medical School.

The results of elmentary analyses and some chemical and physical properties of DNP-amines are listed in Tables I and II. The analytical data on DNP-derivatives of spermidine and spermine show that they are tri- and tetra-DNP compounds, respectively. It is interesting that their molecular extinction coefficients are comparable to that of the di-DNP compound.

TABLE I

Elementary Analyses of DNP-amines

	C (%)		H (%)		N (%)	
	found	calcd.	found	calcd.	found	calcd.
DNP-Ethanolamine	42.53	42.29	4.13	3.99	18.45	18.50
DNP-β-Phenethylamine	58.46	58.53	4.44	4.56	14.57	14.63
DNP-Tryptamine	59.45	58.89	4.66	4.33	17.21	17.17
di-DNP-Tyramine	51.29	51.18	3.32	3.22	15.14	14.92
di-DNP-Histamine	46.08	46.05	3.04	2.96	22.16	22.12
di-DNP-Putrescine	45.80	45.72	3.83	3.84	19.67	20.00
di-DNP-Cadaverine	46.58	47.01	3.93	4.18	19.34	19.35
tri-DNP-Spermidine	46.80	46.66	4.05	3.92	19.84	19.59
tetra-DNP-Spermine	47.31	47.12	4.13	3.95	19.75	19.39
DNP-Agmatine-sulfate[1]	36.64	36.36	5.24	5.27	23.15	23.13

1) Found: S, 4.12; H_2O, 5.15 per cent; DNP-agmatine$\cdot\frac{1}{2}H_2SO_4\cdot H_2O$ theoretically contains S, 4.41; H_2O, 4.96 per cent.

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* This work was presented at the 7th Kinki Local Meeting of the Japanese Biochemical Society held in Osaka in May, 1960.

TABLE II

Some Physical Constants of DNP-amines

	Recrystallization solvent	Melting point (uncorrected) (°C)	Melting point reported (°C)	Absorption maximum in DMFA[2]-water (1 : 1)	Molecular extinction coefficient[1] ($\times 10^{-4}$)	
					at λ max. in DMFA[2]-water (1 : 1)	at 360 mμ in 1% HCl-acetone (3 : 2)
DNP-Methylamine	ethanol	174–175	173.5–175 (*1*) 176–177 (*2*)	360	1.65	1.68
DNP-Ethylamine	〃	114–115	113–114 (*1, 3*)	361	1.72	1.76
DNP-Ethanolamine	aqueous ethanol	88– 89	90 (*4*)	358	1.69	1.71
DNP-*iso*-Butylamine	〃	80– 81	80.2–81 (*1*) 80 (*5*)	361	1.77	1.75
DNP-*iso*-Amylamine	〃	89– 90	91 (*6*)	362	1.80	1.80
DNP-2-Methylbutylamine	—	—		361	1.76	—
DNP-β-Phenethylamine	aqueous ethanol	114–115		356	1.75	1.69
DNP-Tryptamine	aqueous dioxane	215 (d)		362	1.61	1.59
di-DNP-Tyramine	〃	167–168		—	—	1.75
di-DNP-Histamine	〃	208 (d)		357	2.29	1.68
di-DNP-Putrescine	acetone	236–237		359[3]	3.39[3]	—
di-DNP-Cadaverine	dioxane	177–177.5		358	3.00	—
tri-DNP-Spermidine	aqueous DMFA[2]	171		361[3]	3.38[3]	—
tetra-DNP-Spermine	〃	224–225 (d)		363[3]	3.41[3]	—
DNP-Agmatine-sulfate	water	213–213.5 (d)		360	1.48	—

1) E/cm./(mole/liter). 2) N, N-Dimethylformamide. 3) Determined in DMFA.

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The Journal of Biochemistry, Vol. 50, No. 2, 1961

Studies on the Metabolic Function of Biotin

V. Depression of β-Hydroxybutyrate Dehydrogenating Activity in Biotin-Deficient *Bacillus macerans**

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(Received for publication, March 16, 1961)

Recently biotin has been found by Wakil *et al.* (*1*), Lynen *et al.* (*2*) and some other workers to participate in carboxylation and decarboxylation reactions as the prosthetic group of related enzymes. On the other hand there are some reports that suggest biotin does not directly take part in the enzymatic reaction, even though the retardation of the enzymatic activity always occurs under biotin deficient conditions. These are the cases with malic enzyme of *Lactobacillus arabinosus* (*3*) and ornithine transcarbamylase of *Streptococcus lactis* (*4*). In these cases, biotin is assumed to participate in the biosynthesis of their apoenzymes.**

It was previously reported by one of the authors (*6, 7*) that the apparent activities of α-keto acids dehydrogenation by *Bacillus macerans* decreased under biotin deficient conditions and the retarded activity could be restored by the addition of DPN or ATP.*** While, in the course of the experiments with some other dehydrogenases, it was found that when HBA was used as a substrate, the dehydrogenating activity was also reduced remarkably in the biotin-deficient bacteria, but that the retardation could not be restored by the addition of DPN.

The present investigation is concerned with the relation between the HBA-dehydrogenating activity and biotin. The results obtained seem to suggest that biotin does play a role in the biosynthesis of apoenzyme of β-hydroxybutyryl CoA dehydrogenase.

EXPERIMENTALS

Preparation of Cell-free Extracts—The bacteria used throughout this experiment, *Bacillus macerans* Schardinger 3482 (ATCC 3518)†, the culture media and the method of cultivation were the same as those in the previous report (*7*). The amount of biotin was 2,000 mμg. per liter in the normal culture media and 7.5 mμg. per liter in the biotin-deficient one. Normal and biotin-deficient culture attained their maximal growth after the cultivation for 40 and 20 hours, respectively. Then the cells harvested were washed twice with 0.8 per cent NaCl and suspended in *M*/15 phosphate buffer, pH 7.3, and treated in a sonic osccillator for 6 to 10 minutes. After centrifugation at −5°C for 30 minutes at 12,000 r. p. m., the clear supernatant obtained was used as the cell-free preparation.

Analytical Methods—HBA dehydrogenating activity of the preparation was measured by using the several different methods such as manometric method or Thunberg technique or HBA dehydrogenase of rat liver mitochondria. The last method is based on the fact that HBA is dehydrogenated to acetoacetate which accumulates without further transformation in mammalian liver mitochondria. This method was carried out as follows. After incubation of the bacterial extracts with HBA and cofactors in the final volume of 3 ml. for 30 minutes at 30°C, the reaction was stopped by the heat treatment (100°C, 5 minutes) and the mixture was centrifuged. One ml. of the supernatant was then added into 2 ml. of the rat liver mitochondrial suspension (equivalent to 100 mg. of fresh liver), and incubated for 20 minutes at 37°C. Reaction was stopped with 1 ml. of 12% TCA and the mixture was centrifuged. The amount of acetoacetate in 1 ml. of the supernatant was assayed by the method of Walker (*8*). Up to 5 μmoles of HBA in the initial system, linear relation could be seen to hold between the amount of acetoacetate produced and that of HBA added as shown in Fig. 1. Such calibration curve was drawn for each set of the experiment. Rat liver mitochondria was prepared by the method of Schneider (*9*).

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* This work was supported by a grant from the Waksman Foundation of Japan and presented in part before at the Thirty-second Annual Meeting of the Japanese Biochemical Society, Osaka, November 1—3, 1959.

** Review article on the Metabolic Function of Biotin (*5*).

*** Abbreviations used are: DPN, diphosphopyridine nucleotide; TPN, triphosphopyridine nucleotide; ATP, adenosine triphosphate; CoA, coenzyme A; Tris, tris (hydroxymethyl) aminomethane; HBA, β-hydroxybutyric acid; TCA, trichloroacetic acid; P*i*, inorganic phosphoric acid.

† The authors are indebted to Dr. Takeharu Hasegawa, Institute for Fermentation, for the courtesy of furnishing *B. macerans*.

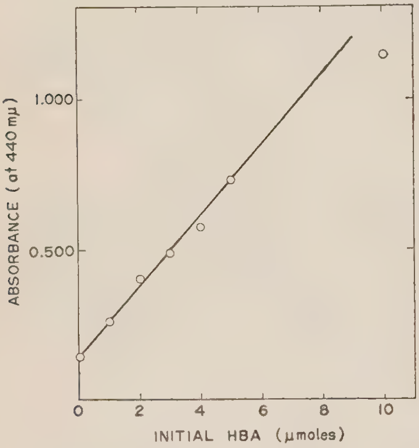

FIG. 1. A standard curve for HBA assay by rat liver mitochondria.

Method and conditions: see the text.

The amount of protein in bacterial extracts was measured turbidimetrically (*10*). Inorganic phosphate determination was carried out by the method of Yoda (*11*). CoA was assayed by the phosphotransacetylase method (*12*). Beckman spectrophotometer model DU was used for all the spectrophotometric measurement.

Materials—ATP was obtained from the Schwarz Bio Research, INC. DPN and TPN were purchased from Sigma Chemical Company. Crude CoA (about 25 per cent) was prepared from the Oriental baker's yeast* following the method of Stadtman and Kornberg (*13*). DL-β-Hydroxybutyric acid was synthesized by oxidation of the corresponding aldol (*14*). Hexokinase was prepared from yeast by Mc Donold's method (*15*).

RESULTS AND DISCUSSIONS

Manometric and Thunberg Experiment—As reported previously (*7*), apparent activities in dehydrogenation of several substrates were observed to be depressed in the biotin-deficient cells and the recovery of these depressions by the addition of DPN were proved manometrically or by the experiments with Thunberg tube. This was shown again by Thunberg technique with the biotin-deficient cell-free extracts. Addition of DPN, however, was proved to be invalid in the case of HBA dehydrogenation (Table I).

TABLE I

Recovery of the Reduced Apparent Dehydrogenase Activities in the Biotin-deficient Cell Extract by DPN

Each vessel contained cell-extract (protein 6 mg.), 20 μmoles of substrate, 1 μmole of DPN, 100 μmoles of phosphate buffer pH 7.5, and 0.5 ml. of 0.01% methylene blue in final volume of 3 ml. Incubation was carried out at 37°C.

Substrate[1]	Decoloration time (min.)	
	−DPN	+DPN
None	60	60
Pyruvic acid	44	4
Malic acid	9	5 1/6
Glutamic acid	13 1/6	7 2/3
DL-HBA	60	60

1) Each acid was added as sodium salt.

Though the normal intact cells showed only faint activity in HBA oxidation by manometric experiment, the extracts from the same cells revealed distinct activity. The further experiments were, therefore, done by using the cell-free system. As seen in Fig. 2, in spite of the addition of DPN, the cell-free

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* The authors are grateful to Mr. Chimaki Shinohara, the Oriental Kôbo KK, for his kind supply of pounds of material.

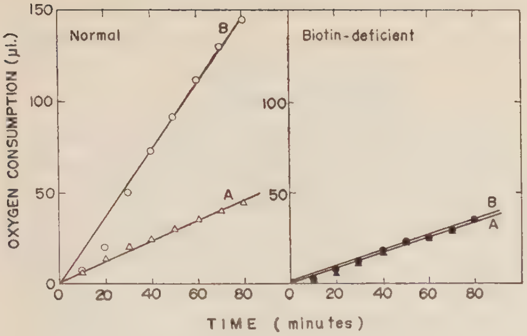

FIG. 2. HBA oxidation by the cell-free extracts.

Each vessel contained extract (protein; normal 22 mg., or deficient 6.7 mg.), yeast hexokinase, 0.1 ml. and the following in μmoles; Pi 0.5, NaF 10, $MgCl_2$ 15, ADP 0.5, DPN 3, glucose 20, and Tris 200 (pH 7.3), in a final volume of 2.5 ml. The side arm contained 40 μmoles of HBA in a volume of 0.5 ml. This was tipped in after equilibration. The center well contained 0.2 ml. of 40% KOH. The reaction was carried out in air at 30°C. A: endogenous, B: +HBA.

extracts of biotin-deficient cells scarcely showed oxygen consumption caused by HBA.

When HBA was used as a substrate the activity of oxidative phosphorylation by these extracts was determined with yeast hexokinase system. Twenty μg. of inorganic phosphate uptake per 15 minutes was observed with normal cell-free extract (P : O ratio was 0.36) but any uptake of inorganic phosphate had never been observed with biotin-deficient cell-free extracts. When pyruvate was added as a substrate, distinct inorganic phosphate uptake was observed by the same preparation of the biotin-deficient extract.

The Decomposition of HBA—From the results of the experiments with manometer and Thunberg tube, it became clear that biotin had some relation to the activity of HBA dehydrogenation. To clarify the participation of biotin in this process of oxidation, the rate of disappearance of HBA was measured by the liver mitochondrial system. In these bacterial cell-free extracts it was proved that part of metabolized HBA was decomposed to acetone and CO_2 through acetoacetate. The results obtained is shown in Table II. Only 0.3 μmoles of HBA in total 5 μmoles were found to be decomposed by the biotin-deficient cell extract while 3.5 μmoles were consumed by the normal one.

TABLE II

Disappearance of HBA in Normal or Biotin-deficient Cell Extract

Reaction mixture contained 10 mg. of protein and the following in μmoles; DL-HBA 10, $MgCl_2$ 4, DPN 2.5, ATP 2.5, CoA 0.05, cysteine 5, and Tris 100, pH 7.3. Incubation was made at 30°C for 30 minutes. Disappearance of HBA was assayed by the rat liver mitochondria (see the analytical methods).

Extract	HBA decreased
Normal	3.5 (μmoles)
Biotin-deficient	0.3

Coenzymes Concerned with HBA Dehydrogenation—D(−) β-Hydroxybutyrate dehydrogenase (*16*) and D(−), and L(+) β-hydroxybutyryl CoA dehydrogenases have been reported to participate in the dehydrogenation of HBA. The first one was found in the animal tissues and is said to transfer hydrogen from HBA to DPN. Among the HBA-CoA dehydrogenases at least two kinds of enzymes have been known which require co-operation of DPN (*17*, *18*) or TPN (*19*) as a coenzyme respectively. The former was found in the animal tissues and the presence of the latter was reported in a *Clostridia*.

The determination of the cofactor requirement of the present enzyme was undertaken by the method of mitochondrial assay. The enzyme was prepared from normal cell extract by twice precipitation with 0.8 saturated ammonium sulfate and then treated with 3% active charcoal. The participation of CoA, ATP and DPN in the present enzyme as cofactors was confirmed (Table III). The effect of free biotin on the enzyme activity was unable to be noticed.

The presence of TPNH oxidase in normal cell extracts was examined and was proved

Table III

Cofactor Requirement of the HBA Dehydrogenating Activity in B. macerans

Incubation and the amount of coenzymes (if added) were the same as those in Table II. Normal cell extract, by ammonium sulfate precipitated and then treated with 3% active charcoal, contained 6.4 mg. of protein. The initial amount of HBA was 10 μmoles.

Addition[1]	HBA decreased
None	0.14 (μmoles)
DPN	0.0
DPN, ATP and CoA	1.35
DPN, ATP, CoA and cysteine	1.62
DPN, ATP, CoA and biotin[2]	1.13

[1] The cofactors were added in the mitochondrial HBA assay system when cell-extract had been incubated without them.

[2] 0.5 μg. of biotin.

to be very weak, so the usual spectrophotometrical experiment was designed for investigation of TPN co-operation. Any increase in absorbance at 340 mμ was not observed by addition of HBA and TPN. Therefore, it seems that TPN is not active as a cofactor of this HBA-CoA dehydrogenase.

These results indicate that the enzyme of *Bacillus macerans* is of a type of DPN-dependent β-hydroxybutyryl CoA dehydrogenase.

Activity of the Enzyme from Biotin-deficient Bacteria—As mentioned above, activity of HBA dehydrogenation in the cell-free extracts of the biotin-deficient bacteria was very weak. The depression could be recovered neither by the addition of biotin nor by the boiled extract of the normal bacteria as shown in Table IV. The existence of any inhibitor in biotin-deficient cell-free extracts was excluded by the experimental results shown in Table V.

Table IV

The Effect of Biotin or the Boiled Normal Cell Extract on HBA Dehydrogenating Activity of the Biotin-deficient Cell Extracts

Incubation systems were the same as those in Table I excepting the amount of the protein (2.6 mg. in exp. 1 and 5.0 mg. in exp. 2).

Experiment no.	Addition	Decoloration time (min.)	
		−HBA	+HBA
1	None	3 1/2	4 1/2
	Boiled ex.	4 1/4	4 1/4
2	None	[1]	43
	Biotin[2]	[1]	64

[1] Did not faded within two hours.

[2] 0.5 μg. of biotin.

Table V

Absence of any Inhibitor of HBA Dehydrogenating Activity in the Biotin-Deficient Cell Extracts

Incubation systems were the same as those in Table I excepting the amount of the protein (5 mg.) in normal cell extract.

Addition	Decoloration time (min.)	
	−HBA	+HBA
None	45	37
Deficient cell-free extract[1]	50	35

[1] The concentration of protein was the same in the normal cell extract.

Apoenzyme Biosynthesis and Biotin—In order to examine whether biotin directly takes part in the reaction or not, an experiment was made on the effect of egg-white, as a source of avidin, on the oxygen uptake by normal cell extracts. But the addition of 0.2 ml. of egg-white with HBA to the extracts did not reduce the rate of oxygen uptake.

Further, the suspension of resting cells of biotin-deficient bacteria was shaken for three hours at 37°C with the mixture of glucose, ATP, DPN, amino acids and with or without biotin. The cells were collected, washed and disrupted by the sonic treatment and the enzyme activities were assayed by the Thunberg method. But significant recovery of the activity was hard to recognize (Table VI).

From these results it may be concluded that biotin participates rather in the biosynthesis of an apoenzyme or apoenzymes which refers to HBA dehydrogenation than in the reaction itself.

TABLE VI

Preincubation of Biotin-deficient Cells with Biotin

Preincubation: Resting cells (55 mg.) in phosphate buffer were shaken with 150 mg. of glucose, 10 mg. of Casamino acids, 5 μmoles of ATP, 2 μmoles of DPN and 1 mg. each of lysine, histidine, tryptophane, phenylalanine, varine, leucine and isoleucine and, when added, 5 μg. of biotin in 300 ml. Erlenmeyer's flask for 3 hours at 28°C.

Assay system: The same as those in Table I excepting the amount of protein (indicated in the table) and methylene blue (0.5 ml. in exp. 1 and 0.1 ml. in exp. 2).

Exp. no.	Biotin	Protein	Decoloration time (min.)	
			−HBA	+HBA
1	−	5.4(mg.)	14 1/4	13 3/4
	−	2.7	28	28
	+	5.0	9 1/6	8
	+	3.7	49	65
2	−	0.5	19 1/6	18 1/2
	+	1.9	14	11 1/2

SUMMARY

1. Under biotin-deficient conditions, *B. macerans* showed only slight activity in β-hydroxybutyrate dehydrogenation.

2. Addition of DPN or other known cofactors were unable to recover the depressed activity in β-hydroxybutyrate dehydrogenation.

3. Experiments with mixed normal and biotin-deficient extracts indicated that the reduced activity was not due to the presence of any inhibitor or to the lack of a heat stable cofactor.

4. HBA dehydrogenating enzyme of this bacteria was determined to belong to β-hydroxybutyryl CoA dehydrogenase which needs DPN as a cofactor.

5. Avidin did not affect the HBA dehydrogenating activity.

6. In this bacteria biotin probably takes a part in the biosynthesis of the apoenzyme(s) which dehydrogenates HBA.

The authors are particularly indebted to Prof. Shozo Tanaka for his discussions and encouragement during this investigation, and also wish to express their thanks to Miss. Tsuya Yoshida for her collaboration.

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The Journal of Biochemistry, Vol. 50, No. 2, 1961

Significance of α-Ketoglutaric Dehydrogenase on the Glutamic Acid Formation in *Brevibacterium flavum*

In the previous papers (*1–8*), it was shown that, in *Brevibacterium flavum* No. 2247, glucose was aerobically metabolized mainly *via* the Embden-Meyerhof pathway, and that C_3-compounds thus formed were further oxidized to form CO_2 and α-ketoglutaric acid *via* the modified TCA cycle with an additional glyoxylate bypass. Since the formation of α-ketoglutaric acid as an end product of the glucose metabolism as well as the apparent inability of this organism to metabolize α-ketoglutarate (*2*) suggested the lack of α-ketoglutaric dehydrogenase in *B. flavum*, α-ketoglutaric dehydrogenase activity as well as related enzyme activities in the bacterial extract was compared with those in the extract of *E. coli* Crookes. Table I clearly shows that the α-ketoglutaric dehydrogenase activity as well as P-enzyme activity in the extract of *B. flavum* is negligibly small, compared to those in the extract of *E. coli*, while the levels of the other enzymes are similar to those in *E. coli* with the exception of isocitritase.

TABLE I

Comparison of Enzyme Activities in B. flavum to Those in E. coli

Enzyme in extract[1]	*B. flavum* No. 2247[2]	*E. coli* Crookes[3]
	μmole/mg. protein/hour	
Glutamic dehydrogenase[4]	1.2	1.2
α-Ketoglutaric dehydrogenase[5]		
(DPN)	<0.01	2.6
,, (TPN)	<0.01	—
P-enzyme[5]	0.016	12.0
Isocitric dehydrogenase[5]	5.3	9.2
Isocitritase[6]	8.8	0.01
Malate synthetase[6]	1.3	1.8

1) Extracted by 0.01 M Tris buffer, pH 7.5, from lyophilized cells.

2) Cells grown in the medium described in the previous paper (*1*).

3) Cells grown in a peptone-meat extracts medium for 24 hours at 30°C.

4) Decrease of reduced triphosphopyridine nucleotide was measured in the presence of α-ketoglutarate and NH_4 salts.

5) and 6) Assay methods used were the same as those described in "Methods in Enzymology" (*8*) and in the previous paper (*9*), respectively.

It is generally accepted that the TCA cycle is the chief pathway of terminal respiration for the most of microorganisms and that it also plays an important role in the synthesis of cell constituents from glucose (*10*, *11*). Therefore, the lack of these enzymes seem to be characteristic of a glutamic acid producing bacterium, *B. flavum*, and to be one of the main causes of the glutamate formation by this organism. Then, a question would arise whether the lack of α-ketoglutaric dehydrogenase equally causes the formation of glutamate in the other bacteria. It has been briefly reported by Davis *et al.* (*12*) that succinate-less mutant 309–1 of *E. coli*, the metabolism of which is blocked between α-ketoglutarate and succinate, was found qualitatively to excrete glutamate aerobically on a succinate-glucose-mineral medium. The formation of glutamate from glucose in *E. coli* mutant 309–1 was further confirmed quantitatively by the present authors in a medium containing only a catalytic amount of succinate (0.1%) in addition to 3.6% of glucose and mineral salts. Namely, 2.3 and 0.0 g. of L-glutamic acid per liter were formed in mutant 309–1 and in *E. coli* W (parent strain), respectively. The results support strongly the above mentioned hypothesis on the mechanism of glutamate formation in certain bacteria.

The effect of α-ketoglutaric dehydrogenase on the isocitritase formation as well as the significance of isocitritase on the glutamate formation was previously discussed (*9*).

The authors are indebted to Prof. B. D. Davis of Harvard Medical School, Boston, for the helpful advices and the gift of *E. coli* mutant 309–1, and to the Institute of Fermentation, Osaka, for the gift of *E. coli* strain W. The authors also thank Dr. H. Oeda, Dr. Y. Tsuchiya, and Mr. T. Tsunoda for their interest and encouragement during the course of this work.

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(Received for publication, May 27, 1961)